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Thank you.

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INTERACTION OF HEAT SHOCK PROTEINS WITH PEPTIDES AND ANTIGEN PRESENTING CELLS: Chaperoning of the Innate and Adaptive Immune Responses

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Key Words dendritic cells, cross-priming, indirect presentation, cancer, infectious diseases

■ **Abstract** Heat shock proteins are abundant soluble intracellular proteins, present in all cells. Members of the heat shock protein family bind peptides including antigenic peptides generated within cells. Heat shock proteins also interact with antigen presenting cells through CD91 and other receptors, eliciting a cascade of events including re-presentation of heat shock protein-chaperoned peptides by MHC, translocation of NFκB into the nuclei and maturation of dendritic cells. These consequences point to a key role of heat shock proteins in fundamental immunological phenomena such as activation of antigen presenting cells, indirect presentation (or cross-priming), and chaperoning of peptides during antigen presentation. Heat shock proteins appear to have been involved in innate immune responses since the emergence of phagocytes in early multicellular organisms and to have been commandeered for adaptive immune responses with the advent of specificity. These properties of heat shock proteins also allow them to be used for immunotherapy of cancers and infections in novel ways.

THE MHC AND THE HEAT SHOCK PROTEINS, A COMMON PEDIGREE

Transplantation of tissues and tumors among mice led to the identification of fundamental immunological roles for two major groups of molecules, the MHC and the heat shock proteins (HSPs). At first sight, the HSPs and the MHC proteins appear quite dissimilar. The MHC proteins are of very recent evolutionary vintage, while the HSPs appeared at the very dawn of life. The MHC are among the most polymorphic (poly-allelic) proteins, while the HSPs are typically monoallelic. The MHC are cell surface proteins, whereas the HSPs are essentially intracellular. The MHC are expressed at modest levels while the HSPs are embarrassingly abundant. However,

0732-0582/02/0407-0395$14.00

these significant differences hide powerful similarities and convergence of functions. In fact, the HSPs have been among the key players in host defense for billenia and appear to have been laying the groundwork for many of the latter day functions of the MHC molecules. I aim to tell here the story of the primordial functions of the HSPs in innate immunity and to describe the many paths where the functions of the MHC proteins and the HSPs converge to create the symphony of adaptive immunity.

While the MHC molecules are familiar to immunologists, a brief introduction to HSPs may not be out of place (1). Approximately 40 years ago, somebody inadvertently turned up the temperature of an incubator full of fruit flies, and the salivary gland chromosomes of the fruit flies, thus heat-shocked, showed the characteristic puffs indicative of transcriptional activity at discrete loci (2). These loci came to be known to encode HSPs, which were gradually identified in all species tested. They are expressed in all cells in all forms of life and in a variety of intracellular locations: in the cytosol of prokaryotes and in the cytosol, nuclei, endoplasmic reticulum, mitochondria, and chloroplasts of eukaryotes. In addition to their ubiquity, the HSPs constitute the single most abundant group of proteins inside cells. They are expressed in vast quantities under normal non–heat shocked conditions, and their expression can be powerfully induced to much higher levels as a result of heat shock or other forms of stress, including exposure to toxins, oxidative stress, glucose deprivation, etc. Approximately ten families of HSPs are known, and each family consists of anywhere from 1 to 5 closely related proteins. There is little or no obvious homology among the individual HSP families even as members within a family are closely related. All HSP families are represented in all organisms although individual members may show variety in distribution. Since their discovery, an increasing array of functions such as folding and unfolding of proteins (3), degradation of proteins (4), assembly of multi-subunit complexes (5), thermotolerance (6), buffering of expression of mutations (7), and others have been attributed to HSPs. In addition, they have become absorbing models for the study of transcriptional regulation (8), stress response (6), and evolution (9).

In order to tell the story of the common experimental pedigree of the MHC and the HSPs, I begin with a quote from a recent article by George Klein that summarizes the beginnings of the connection between the MHC molecules and tumor immunity (10):

> During the first part of the 20th century, cancer researchers spoke about transplantable and non-transplantable tumors. The mice and rats were not inbred and transplantability meant therefore transgression of histocompatibility barriers, but most researchers were unaware of this . . . tumor immunology was an optimistic field due to this artifact. In spite of this clear, definitive evidence that was available already in the early 50s, the artifactual, allograft-based "tumor immunology" continued to flourish during at least one more decade. Meanwhile Ludwik Gross performed some not too well controlled experiments suggesting that chemically induced mouse sarcomas could be immunogenic in syngeneic mice. Subsequently Prehn and Main confirmed this in critically controlled experiments . . . Their data also indicated that the chemically

induced tumors did not cross-react with each other . . . We suspected that even the experiments of Prehn and Main may have been flawed. Obviously, the ultimate evidence had to be based on experiments with the primary, autochthonous tumor host. We did these rather laborious experiments and published them in 1960 in *Cancer Research*. Yes, it was all true.

Figure 1 shows the general outline of the experiments of Klein et al. (11). They demonstrated the extraordinary phenomenon that one could immunize against

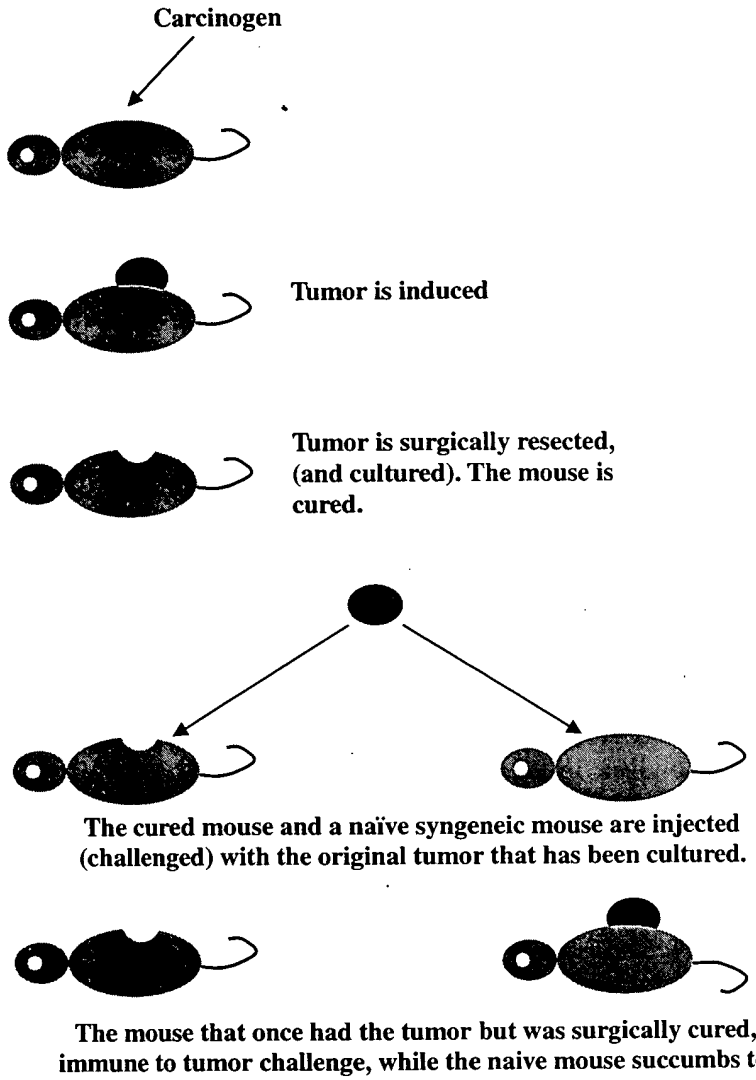

Figure 1 A cartoon showing the design of the experiments of Klein et al. (11) that established formally that methylcholanthrene-induced tumors are immunogenic in the primary and in the syngeneic hosts, and that immunity is individually tumor-specific.

syngeneic tumors in the same manner as one could against smallpox and polio viruses. The extraordinariness derived from the fact that in contrast to viruses, the tumors were of self origin and yet were immunogenic. Inherent in this observation was the prediction that the tumors express tumor-specific antigens. Further, as the individual tumors were not cross-reactive, the tumor-specific antigens were individually rather than commonly tumor-specific. Speaking with respect to the identity of these antigens, referred to at the time as tumor-specific transplantation antigens (TSTAs), Klein ends his article with the accurate remark "The TSTAs of the chemically induced tumors are still a mystery."

I began to look for the cancer-specific antigens by their ability to elicit protective immunity to cancer challenges, i.e., by the very assay that pointed to their existence (see 12). This approach typically involved fractionation of cancer homogenates into various protein components by conventional chromatographic methods. The fractions thus obtained were used to immunize animals that were then challenged with live cancer cells. The fractions that elicited protection against the cancer were then re-fractionated and the cancer rejection assay repeated until apparently homogeneous preparations were obtained. This approach, with variations, led to identification of cancer-rejection molecules from cancers of diverse histological origins, induced in mice and rats of different haplotypes by chemicals or UV-radiation, or they were of spontaneous origin (Table 1). The cancers ranged in immunogenicity from the nonimmunogenic (e.g., the Lewis lung carcinoma) to the highly immunogenic regressor cancers induced by UV-radiation. Surprisingly, all the well-characterized molecules identified by this method, by us and then by others, turned out to be HSPs of the hsp90, hsp70, calreticulin, or the grp170 family (Table 1).

The phenomenon of graft and tumor rejection among histo-incompatble mice played a key role in the discovery of histocompatibility and the molecules that mediate it, i.e., the MHC molecules. The same phenomenon in histocompatible (syngeneic) mice led to the discovery of the molecules that mediate such rejection, i.e., the HSPs.

THE STRANGE IMMUNOGENETICITY OF VERY COMMON MOLECULES: DISCOVERY OF HSP-ASSOCIATED PEPTIDES

Consistent with the experiments with intact tumors, the HSPs purified from a given cancer were observed to elicit protective immunity specific to that particular cancer. HSPs derived from normal tissues did not elicit protective immunity to any cancers tested (13). The observed specificity of immunogenicity of cancer-derived HSPs suggested that HSPs ought to harbor somatic polymorphisms, such that HSPs would differ between cancers and normal tissues and from one cancer to another. However, extensive sequencing studies of HSP cDNAs of cancers and normal tissues did not support that idea (14). What then was the basis of the specificity of immunogenicity of these very common HSP molecules? The first

TABLE 1 Representative studies that demonstrate the tumor-specific immunoprotective activity of tumor-derived HSP-peptide complexes, or infectious disease-specific immunoprotective activity of HSP-peptide complexes derived from infected cells. Immunogenicity of tumors is graded as $^{-}$,$^{+}$,$^{++}$ or $^{+++}$ by subjective criteria. Asterisks refer to models of therapy of pre-existing cancers, as opposed to models of prophylaxis.

Cancer or infectious agent	Induced by	Immunogenicity	Host	Molecule	Ref.
Zajdela hepatoma	Chemical	++	Rat	gp96	39
Meth A fibrosarcoma	Chemical	++	BALB/c mice	gp96	50, 68*, 94*
				hsp90	98
				hsp70	18
				hsp110	99
				grp170	99
CMS5	Chemical	+	BALB/c mice	gp96	50
CMS13	Chemical	++	BALB/c mice	gp96	100
Lewis lung ca.	Spontaneous	-	C57BL/6 mice	gp96	68*, 94*
				hsp70	68*
B16 melanoma	Spontaneous	-	C57BL/6 mice	gp96	68*, 101*
CT26 colon ca.	Chemical	++	BALB/c mice	gp96	68*
Colon 26 Ca.			BALB/c mice	hsp110	99*
				grp170	99
UV6138	UV	+++	C3H mice	gp96	102
UV6139SJ	UV	++	C3H mice	gp96	68*, 102
Dunning G prostate ca.		+	Rat	gp96	103*
A20 B cell lymphoma		+	BALB/c mice	gp96	104
				hsp70	104
				hsp90	104
				Calreticulin	104
L15/0 lymphoma	Spontaneous	+	Xenopus	Gp96	90
				Hsp70	90
M. tuberculosis			BALB/c mice	Gp96	22
Listeria			BALB/c mice	Gp96	22
LCMV			BALB/c mice	Hsp70	105

obvious answer lay in the possibility that the homogeneous HSP preparations were not so homogeneous after all, but contained unexamined contaminants that were responsible for the immunogenicity. This possibility, as sensible as it was depressing, did not turn out to be true: The immunogenic HSP preparations were certifiably free of other protein contaminants as determined by all structural criteria tested, and the immunogenicity did not derive from associated carbohydrates, lipids, or nuclei acids (P. Srivastava, unpublished observations). The possibility was then envisaged that low molecular weight substances, not detectable by polyacrylamide gel electrophoresis, are associated with HSPs and are responsible for the specificity of immunogenicity of HSP preparations (15, 16). This idea was tested and

derived a modicum of support when a large collection of peptides could be shown to elute from a homogeneous gp96 preparation as it was treated with trifluoroacetic acid (17). Strong support for the idea came when treatment of an immunogenic (tumor-protective) hsp70 preparation with ATP had two consequences: It resulted in elution of a wide array of peptide peaks from the hsp70 polypeptide, leaving the polypeptide intact, and it rendered the hsp70 preparation nonimmunogenic, i.e., ineffective in immunizing against cancer cells, even though the hsp70 polypeptide was present in equivalent amounts in untreated and ATP-treated hsp70 preparations (18). This was the first demonstration that hsp70, as isolated from tumors, was associated with peptides, that dissociation of peptides from hsp70 resulted in abrogation of the immunogenicity, and that the hsp70 polypeptide was not immunogenic in and of itself. It was shown subsequently that the hsp70 and gp96 are associated with peptides in vivo, and the observed association of hsp70 and gp96 with peptides is not the result of an artifact occurring after cell lysis and during purification of the HSPs (19). Considerable immunological and structural evidence now supports the notion that certain HSP molecules (gp96, hsp90, hsp70, calreticulin, hsp110, and grp170) are peptide-binding proteins and are associated with antigenic epitopes (Tables 1, 2).

The Immunological Evidence

The immunological evidence for association of HSPs and antigenic peptides has continued to accumulate at an impressive pace. The large number of studies that

TABLE 2 Selected structural or immunological studies that have shown that specific, defined antigenic peptides are associated with HSPs

Epitope/antigen	MHC I	Ref.
TUMOR ANTIGENS		
PRL1a mouse leukemia	L^d	28
Human melanoma MART-1	A2	26
Human melanoma tyrosinase	A2	26
Human melanoma gp100	A2	26
VIRAL ANTIGENS		
Vesicular stomatitis	K^b	23, 27
Herpes simplex-2	d	25
Influenza	K^b	20
SV40	D^b, K^b	106
Hepatitis B ag		30
MODEL ANTIGENS		
β galactosidase	L^d	24
Ovalbumin	K^b	29
NORMAL CELLULAR ANTIGENS		
Minor H	K^d, K^b	24

show the individually tumor-specific immunogenicity of tumor-derived gp96, hsp70, hsp90, calreticulin, hsp110, and grp170 preparations has been referred to earlier (Table 1). When the hypothesis that HSPs must associate with various cellular antigens was first proposed (15, 16), it was argued broadly that HSP preparations purified from cells infected with viruses or other infectious agents must be associated with the antigenic epitopes of those agents and that such HSP-peptide complexes should be found to be immunoprotective against the cognate infectious agents (Table 1). That prediction has been amply fulfilled. Gp96 preparations isolated from influenza virus–infected cells have been shown to be protective against a challenge with the influenza virus (20, 21). Gp96 preparations isolated from mouse tissues infected with *Mycobacterium tuberculosis* and *Listeria* are protective specifically against those agents (22).

A dramatic demonstration of the binding of endogenously generated antigenic peptides to gp96 came from the work of Podack and colleagues (22a), who constructed a gene encoding a gp96 molecule fused with the Fc portion of murine IgG1, generating a secretory gp96-Ig. Transfection of gp96-Ig into tumor cells decreased their tumorigenicity and increased their specific immunogenicity. The tumors were rejected after initial growth. In addition to demonstrating the binding of peptides to gp96 in vivo, these studies provide a common tool for easy generation of gp96-peptide complexes for any tumor, and possibly also for immunotherapy of human cancer. Zheng et al. (22b) have shown the broader applicability of this idea and have further developed it mechanistically, as discussed in another section.

These studies do not provide definition of the antigenic epitopes associated with the HSPs, but they provide compelling circumstantial evidence by virtue of the fact that HSPs purified from antigen-negative control cells did not immunize against the particular tumor, virus, or parasite. A number of other studies provide direct evidence of association of defined antigenic epitopes with HSP molecules (Table 2). Gp96 preparations isolated from vescicular stomatitis virus (VSV)–infected or SV40-transformed cells elicit classical MHC I–restricted, antigen-specific cytotoxic T lymphocytes (CTLs) against defined antigenic epitopes of the two viruses (20, 23). In experiments with VSV, the gp96 preparations were able to cross-prime; preparations from VSV-infected cells of the *b* or the *d* haplotypes could immunize mice of the *b* haplotype and elicit *b*-specific CTLs, thus showing that gp96 was associated with peptides regardless of the MHC I haplotype of the cells from which it was purified. Arnold et al. (24) showed that immunization with gp96 preparations isolated from cells transfected with the gene encoding β-galactosidase elicited CTLs specific for an L^d-restricted epitope of β-galactosidase; similarly, immunization with gp96 preparations purified from cells expressing selected minor histocompatibility antigens was able to prime (as well as cross-prime) CTL responses against the particular minor antigens. More recently, Navaratnam et al. (25) immunized mice with gp96 isolated from cells transfected with the gD antigen of Herpes simplex virus-2.

The first evidence of association of antigenic peptides with human HSPs comes from a recent study by Castelli et al. (26) who showed that human

melanoma-derived hsp70, but not hsp70 from other human sources, was associated with peptides corresponding to antigenic epitopes derived from gp100, Mart 1, and tyrosinase, but not Trp2. Issels and colleagues have obtained similar results with the chaperoning of tyrosinase epitopes associated with hsp70 isolated from a human melanoma (personal communication). Corresponding studies with gp96 from human melanoma cells are now in progress (C. Castelli, G. Parmiani, personal communication).

The Structural Evidence

What are the structural characteristics of the HSP-associated peptides? Immunological evidence indicates that HSPs appear to bind all peptides tested. In 13/14 instances in which the association of a given antigenic epitope with HSPs has been sought, such association has been found. (Trp2 is the sole exception thus far, and the basis of its absence in human melanoma-derived hsp70 preparations is unclear.) Based on the ability of HSP-peptide complexes to cross-prime, HSPs associate with peptides regardless of the MHC haplotype of the cells from which they are isolated. These lines of evidence point to a promiscuous ability of HSPs to bind peptides. Such promiscuity is consistent with the primordial roles of HSPs in folding and assembly of proteins, and it requires structural definition. In spite of an impressive number of studies reporting the presence of HSP-associated peptides as detected immunologically in a diverse array of systems (Table 1, 2), structural scrutiny of HSP-associated peptides has been more limited (Table 2). Four studies to date have analyzed HSP-associated peptides structurally (27–30), and each has done so with respect to a single peptide. Nieland et al. (27) first identified a known K^b-restricted viral epitope to be associated with gp96 purified from virus-infected cells; such peptides could not be detected in gp96 preparations from uninfected cells. Consistent with the cross-priming studies described earlier, the epitope was detected in VSV-infected cells of the *b* or the *d* haplotype. In a study with a mouse leukemia (28), whose L^d-restricted epitope has been defined, Ishii et al. isolated gp96, hsp90, and hsp70 from the leukemic cells. They eluted peptides from each of the three preparations and fractionated them by column chromatography. Each column fraction was tested for the presence of the antigenic epitope by pulsing L^d-expressing antigen-negative cells with it and testing the ability of specific CTLs to lyse them. Antigen-positive factions were identified among peptides eluted from each of the HSP preparations and were analyzed by mass spectroscopy. Interestingly, while each of the HSPs was associated with the precise epitope, hsp90 and gp96 were found to be associated in addition with longer precursor peptides of it. Breoler et al. (29) identified the ovalbumin-derived SIINFEKL epitope associated with gp96 and hsp70 isolated from ovalbumin-transfected cells. Meng and colleagues (30) have provided the first structural evidence for association of antigenic peptides with a human HSP. They isolated gp96 from human livers infected with hepatitis B virus and showed, by mass spectroscopy, the presence of a virus-encoded peptide with it.

HSP-associated peptides have not been examined thus far with the tools that have been so effective in corresponding analyses of MHC-associated peptides. Attempts in this direction have often floundered partly because the HSP molecules themselves have often degraded during elution of peptides, as a result of trace amounts of proteases present in gp96 preparations used (H-J. Schild, personal communication). However, improvements in the methods of purification used as well as the use of increasingly sophisticated mass spectroscopy tools is beginning to address this lacuna (C. Liu, P.K. Srivastava, unpublished observations). Such studies must be distinguished from others that have characterized the peptides that may be made to associate with HSPs in vitro (31, 32). Using such assays in vitro, Flynn et al. (31) suggested that "the peptide-binding site of hsp70 selects for aliphatic residues and accommodates them in an environment energetically equivalent to the interior of a folded protein." Blond-Elguindi et al. (32) have suggested a sequence motif for peptides that may bind BiP in vitro. However, this motif is at variance with other studies that have shown hsp70-peptide binding. Blachere et al. (33) and Basu & Srivastava (34) have also analyzed a number of peptides for binding gp96, hsp70, and calreticulin in vitro and have observed considerable variation among peptides with respect to their ability to bind the HSPs. Clearly, more studies, modeled on corresponding studies with the MHC molecules, are needed to resolve the questions.

The evidence for structural features of HSPs that allow them to bind peptides may be described as following. Zhu et al. (35) crystallized a ligand-binding fragment of the bacterial hsp70 known as DnaK and identified a definite peptide-binding pocket in it. "The structure consists of a beta-sandwich subdomain followed by alpha-helical segments. The peptide is bound to DnaK in an extended conformation through a channel defined by loops from the beta sandwich." The peptide-binding activity of gp96, hsp70, and calreticulin has been demonstrated independently by Blachere et al. (33), Wearsch & Nicchitta (36), Basu & Srivastava (34) and Sastry et al. (37, 38). Wearsch et al. (36) have used fluorescent probes to identify the presence of a possible hydrophobic peptide-binding pocket in gp96. Pursuing a similar theme, Sastry et al. (37, 38) have used peptides tagged with fluorescent probes to explore the molecular environment of the peptide-binding site of gp96. Based on these studies, they have identified the amino acid position 624-630 in a highly conserved region of gp96 as the peptide-binding site (38).

While these results are potentially illuminating, the not-too-well-controlled use of a large bulky probe that may alter the physicochemical properties of gp96-peptide interaction places some doubt on their general validity. Interestingly, Nicchitta and colleagues as well as Sastry suggest that gp96 molecules exist as dimers and that the dimeric state is the true peptide-binding state. These observations recapitulate the original observations of Srivastava & Das (39), who demonstrated that gp96 (then called p100) molecules eluted from size exclusion columns exclusively as dimers and tetramers. The cytosolic homologue of gp96, hsp90, is also being studied structurally. Scheibel et al. (40) suggest the existence of two substrate-binding sites in hsp90, while ongoing crystal structure studies with hsp90

are in the process of clarifying the identity and structure of the peptide-binding pocket of hsp90 (41–43). Buchner and colleagues (44) suggest that the hsp90 contains two and perhaps more distinct peptide-binding sites.

The thesis that HSP molecules are associated with peptides including antigenic peptides was advanced solely to explain the specific immunogenicity of tumor-derived homogeneous HSP preparations. Ten years later, there is overwhelming evidence for that proposition. Tumor antigens, viral antigens, antigens of intracellular parasites, mouse antigens, human antigens, cytosolic antigens, nuclear antigens, and secreted antigens have all been shown to be associated with the HSPs, and the peptide-binding pocket of at least one of the HSPs has been defined through crystallographic analysis. However, similar studies with other HSPs are yet to be carried out. The rules through which apparently any peptides are able to bind the HSPs have yet to be defined. These are decidedly rewarding avenues for future structural analyses, and the elegant and extensive work carried out with MHC-associated peptides provides a powerful precedent.

"The TSTAs of the Chemically Induced Tumors Are Still a Mystery"

Let us return briefly to the initial question regarding the identity of the antigenic peptides that confer individually specific immunogenicity upon tumors. Such immunoprotective peptides have been identified in a small number of instances (see 45), and in each instance, they are mutations of normal proteins. There is no common pattern among the mutations identified, and I believe that the immunogenicity is a consequence of the random mutations that are an inevitable part of cell division (14). The individually unique antigenicity of tumors suggests a lack of relationship between the transforming and the immunogenic mutations. Unique, individually tumor-specific antigens resulting from random mutations are being increasingly identified in human cancers as well (46). With respect to the present context, the immunogenic antigens of tumors are associated with HSPs purified from the tumors in the two instances tested, i.e., a mouse leukemia (26) and a fibrosarcoma (T. Matsutake, P.K. Srivastava, submitted). Should the proposal (14) that random mutations not associated with malignant transformation are the basis of immunogenicity of mouse (and human) cancers continue to be substantiated, the mystery of TSTAs will have been resolved not in favor of an instantly gratifying molecule or family of molecules but as a myriad mutations, random but specific, in the common molecules of the cancer proteome.

HSPs Are Adjuvants: HSP-Peptide Complexes Elicit $CD8^+$ T Cell Responses in Spite of Exogenous Administration

The unequivocal demonstration that the specific immunogenicity of tumor-derived HSP preparations is elicited by HSP-associated peptides leads inevitably to the question of whether immunogenic HSP-peptide complexes could be generated in vitro. Blachere et al. (33) did just that. They reconstituted gp96-peptide and hsp70-peptide complexes in vitro using a panel of 7 peptides, and they showed

that while HSPs alone and peptides alone were nonimmunogenic, HSP-peptide complexes elicited MHC I–restricted antigen-specific $CD8^+$ CTLs. These results were reproduced by Houghton (Moroi, et al. 47) who demonstrated that BiP-peptide complexes were similarly immunogenic. These authors modified the original approach of Blachere et al. (33) by using a peptide that contained the antigenic epitope and another sequence selected for a higher affinity binding to BiP. There was little evidence that the higher affinity interaction resulted in significantly higher immunogenicity over that of the unmodified peptide; however, this is an interesting question that will no doubt be resolved through future experiments. In another variation of the original approach, Suzue et al. (48) fused a mycobacterial hsp70 gene with a fragment of the ovalbumin gene and purified the fusion product. Immunization with the fusion product led to potent ovalbumin-specific CTLs and rejection of an ovalbumin-expressing tumor. A number of other genes, such as those encoding papilloma virus or malarial parasite antigens have since been fused with hsp70, and the immunogenicity of such fusion products has been demonstrated (49, 49a).

In addition to settling unequivocally the questions for which they were intended, the experiments of Blachere et al. (33) led to a number of other significant findings and implications. First, they showed that HSPs could be loaded in vitro with synthetic peptides. For this, the HSPs could first be denuded of associated peptides (as by ATP treatment in case of hsp70), or they could be gently denatured in the presence of a higher temperature (50°C) or of guanidium hydrochloride (as in case of gp96) and then renatured in the presence of exogenous peptides. Either treatment led to association of the exogenous peptides with the HSPs. The extent of reconstitution was variable, and depending upon the HSP and the conditions used, between 1% and 10% of gp96 molecules could be loaded with peptides.

Second, they showed, remarkably, that the HSP-peptide complexes were stable under conditions of denaturing polyacrylamide gel electrophoresis. Unlabeled HSPs complexed with labeled peptides migrated as radioactive bands of the size expected of the HSP-peptide complexes. This observation is consistent with our results in 1986 where a gp96 band eluted from denaturing gels was used to immunize mice successfully against the tumor from which gp96 was isolated (50). There is little precedent for this kind of noncovalent interaction, and it highlights the need for close structural examination of HSP-peptide interaction as discussed in the previous section.

Third, the results of Blachere et al. show that HSP-peptide complexes elicit $CD8^+$ T cell responses in spite of exogenous administration. Exogenous antigens are typically routed through the MHC II–presentation pathway and elicit $CD4^+$ responses, whereas endogenously synthesized antigens are presented through MHC I molecules and stimulate $CD8^+$ cells (51). In only a small number of instances have exogenous antigens been shown to enter the MHC I–presentation pathway (52). This demonstration makes HSPs powerful adjuvants for generation of $CD8^+$ responses and makes them the first adjuvants of mammalian origin. (See a later section for discussion of adjuvanticity of α2 macroglobulin or α2M.) In this

regard, we have recently made the not-too-surprising observation that immunization with HSP-peptide complexes also elicits antigen-specific MHC II–restricted $CD4^+$ response (T. Matsutake and P. Srivastava, submitted for publication).

Fourth, Blachere et al. (33) demonstrated that the quantity of peptide complexed to HSP molecules required for successful immunization is extremely small. As little as a few hundred picograms to a nanogram of peptide, if complexed to an HSP, was found to be sufficient to immunize. This observation, which is also germane for the adjuvanticity of the HSPs, brought into sharp focus the novelty of the mechanism of specific immunogenicity of HSP-peptide complexes (discussed in the next section). Finally, Blachere et al. (33) showed that immunogenicity did not result when peptides were complexed with mouse serum albumin that binds peptides just as effectively as the HSPs do. This suggested that the HSPs were doing something more than simply protecting the peptides from degradation or other such physical dangers. This observation too had a powerful role in our imagining of the mechanism of immunogenicity of HSP-peptide complexes.

MECHANISMS OF IMMUNOGENICITY OF HSP-PEPTIDE COMPLEXES: THE EIGHT-FOLD PATH THROUGH THE HSP RECEPTORS

The Two Paths ($CD8^+$ and $CD4^+$) to Adaptive Immunity

We were impressed with the fact that immunization with femtomole quantities of antigenic peptides chaperoned by HSPs (but not other proteins) was effective in eliciting such potent T cell responses (33). At the same time we had learned that priming of immune response by HSP-peptide complexes was exquisitely sensitive to abrogation of function of antigen presenting cells (APCs) (53). Putting these ideas together with the general biological principle that extraordinary efficiencies are often achieved through specific receptors, we proposed that HSPs interact with APCs through specific receptors and that such interaction results in endocytosis of HSP-peptide complexes followed by processing of peptides and their presentation by MHC I molecules (54).

The first step in validation of this idea came from experiments that showed that macrophage, but not B cells or fibroblasts, take up gp96-peptide complexes (isolated from cells or reconstituted in vitro) and re-present the gp96-chaperoned peptides on the MHC I molecules of the macrophages; re-presentation does not occur by transfer of peptides from the gp96 molecules to MHC I on the cell surface but does require internal processing (23). Singh-Jasuja et al. (55) further demonstrated that receptor-mediated endocytosis of the gp96-chaperoned peptides is essential for re-presentation of these peptides by MHC I; nonspecific endocytosis of the gp96-peptides does not result in re-presentation. Essentially similar data for re-presentation of hsp70-chaperoned peptides were shown recently by Castellino et al. (56). The extraordinary efficiency of the process observed earlier through immunization experiments became evident again through such re-presentation

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assays. Gp96-chaperoned peptides were re-presented by the MHC I molecules of the APC several hundred-fold more efficiently than unchaperoned peptides.

Subsequently, we and others demonstrated specific, saturable, and competitive binding of various HSPs to APCs (57–59) as further evidence for the existence of an HSP receptor on APCs. In attempting to identify the long-proposed HSP receptor (54), Binder et al. (60) applied solubilized membranes of APCs on gp96 affinity columns and eluted and sequenced a gp96-binding protein. This turned out to be the previously known $\alpha 2$ macroglobulin ($\alpha 2$M) receptor CD91. $\alpha 2$M as well as antibodies to CD91 were shown to inhibit completely the re-presentation of gp96-chaperoned peptides by APCs. Soon thereafter, Basu et al. (61) demonstrated that CD91 acted as the receptor not only for gp96 but also for hsp90, hsp70, and calreticulin. These data were interpreted to suggest CD91 as a global HSP receptor on APCs. The wider significance of this observation is discussed later under its own heading, and we shall leave the subject of HSP receptors for now.

Basu et al. (61) also shed some light on the pathway of intracellular processing of gp96-chaperoned peptides (Figure 2). It was clear from the work of Arnold et al. that the endocytosed gp96-peptide complexes enter an endosomal compartment (57) and from the work of Suto & Srivastava (23) that these compartments were not acidic. Basu et al. (61) showed that further processing of peptides required a functional proteasome and transport of the peptides through transporter associated with antigen processing (TAP), followed by the classical secretory pathway. This picture appears reasonable and straightforward. However, the mechanism of transport of peptide from the endosome to the cytosol is unclear. Further, Castellino et al. (56), who have shown an essentially similar pathway for re-presentation of hsp70-chaperoned peptides, have made the additional and remarkable observation that the structure of the peptide can dictate if the transport of the peptide into the ER is TAP-dependent or not. Thus, while the broad outlines of the internal trafficking of HSP-chaperoned peptides from the APC surface to binding to the MHC I molecules of the APCs are clear, a number of interesting and important details remain to be characterized.

We have demonstrated recently that the HSP-chaperoned peptides are re-presented by the MHC II molecules of the APCs, in addition to re-presentation by MHC I molecules discussed thus far. Presentation of the exogenous antigens through the MHC II molecules of APCs is not surprising in and of itself. What is surprising here is the observation that the re-presentation by MHC II molecules also occurs through the CD91 receptor and that in quantitative terms, it is significantly more efficient than re-presentation through phagocytosis. This observation indicates that once an HSP-peptide complex is taken up through CD91, it may enter one or more of several trafficking and processing pathways. The factors that contribute to such molecular decisions would make for important discoveries not only for the biology of the APCs, but for cell biology in general. They will also have significant implications for strategies of vaccination.

Some number crunching will illustrate how such a small quantity of antigenic peptide complexed with a small quantity of a HSP becomes powerfully immunogenic. Typically, a mouse is immunized with 1 μg of HSP-peptide

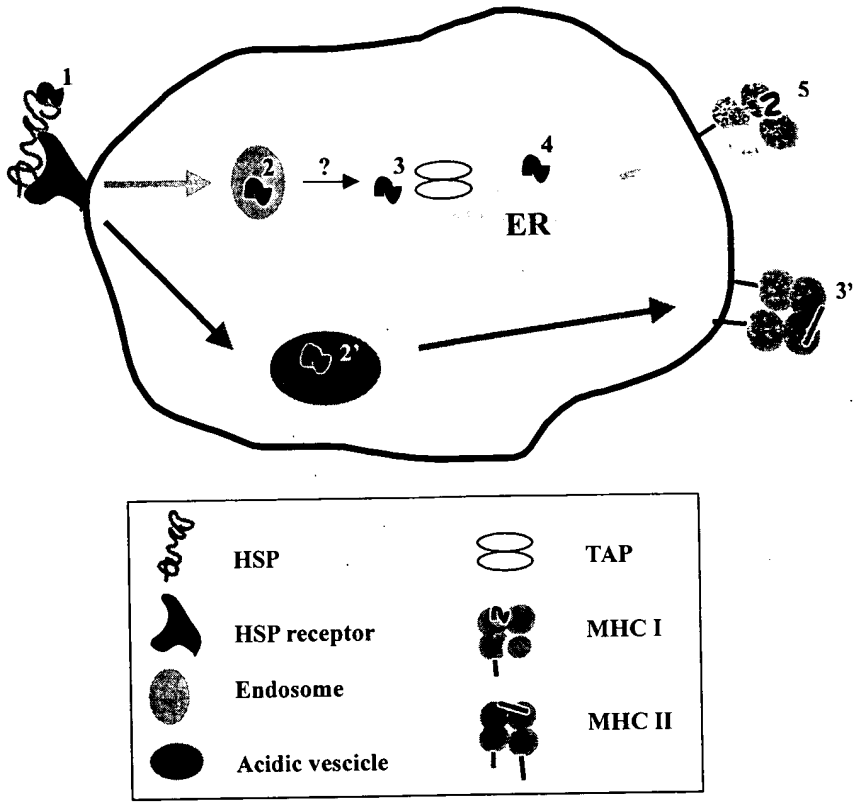
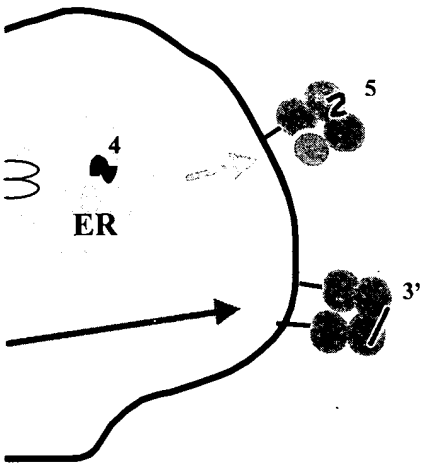
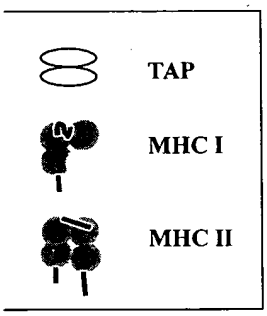


Figure 2 The pathways of re-presentation of HSP-chaperoned peptides by MHC I and MHC II molecules of the APCs (macrophages and DCs) after uptake of the HSP-peptide complexes through CD91 molecules. All HSPs tested use the CD91 receptor for both pathways. The re-presentation of peptides by MHC I requires proteasomal activity and TAP (61), although TAP-independent mechanisms may also operate (56).

complexes intradermally in order to get full tumor protection. In case of gp96, this is equivalent to $\sim 6 \times 10^{12}$ gp96 molecules chaperoning perhaps half as many peptides (assuming that the dimer is the minimum peptide-binding unit). The proportion of specific peptides among these may be estimated conservatively as 1/100,000 (in absolute numbers, 3×10^7 specific peptides in the immunizing dose). The immunization is carried out intradermally in an area of <0.1 cm^2 harboring $\sim 10^4$ Langerhans cells. One may imagine these 3×10^7 antigenic peptides to be delivered into the MHC I and MHC II presentation pathways of $\sim 10^4$ Langerhans cells, presenting on an average of $\sim$3000 peptides on their MHC I and/or MHC II molecules. These cells migrate to the lymph nodes, as we have shown (62), and then stimulate the naive T lymphocytes. Ten thousand activated, mature dendritic cells (DCs) presenting 3000 MHC-peptide complexes each are a powerful





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immunological stimulus; indeed even one tenth as many DCs presenting one tenth as many antigenic peptides are powerful enough to elicit a potent T cell response.

The Six Paths to Innate Immunity

Re-presentation of HSP-chaperoned peptides by MHC molecules was first proposed and then experimentally demonstrated as a mechanism to explain the specific immunogenicity of HSP-peptide complexes. Our attention was exclusively on specificity, and we were thus surprised to find that exposure of APCs to gp96 (or other HSPs) led to secretion of low levels of TNFα by the APCs, regardless of whether or not the gp96 molecules were associated with the antigenic peptide (23). As the levels were low, and the results not consistent with our inclination, we initially attributed them to background or to contaminating lipopolysaccharides (LPS) in our HSP preparations or buffers. However, the low levels persisted reproducibly and at first look appeared unrelated to LPS contamination. This led us to a full-scale analysis of the phenomenon, the most difficult aspect of which was to convince ourselves that it was not a result of LPS contamination. With time and effort, we were able to do so and, with considerably more time and effort, were even able to publish the findings. In these reports, Basu et al. (63), Binder et al. (62), and Panjwani et al. (64) showed through in vitro and in vivo studies that the interaction of HSPs gp96, hsp90, and hsp70 with APCs led to a series of events associated with innate immunity. Singh-Jasuja et al. (65), Chen et al. (66), and Asea et al. (67) also came to partially overlapping conclusions with gp96, hsp60, and hsp70, respectively.

The innate immune responses set in motion by the HSP-APC interaction may be summarized as follows:

1. Secretion of inflammatory cytokines TNFα, IL-1β, IL-12, and GM-CSF by macrophages (63). The IL-12 thus secreted acts to stimulate proliferation of NK cells (68);
2. secretion of chemokines such as MCP-1, MIP-2, and RANTES by macrophages (64, 69) and possibly by T cells (69);
3. induction of inducible nitric oxide synthase and production of nitric oxide by macrophages and DCs (64a);
4. maturation of DCs as measured by enhanced expression of MHC II, B7-2, and CD40 molecules on CD11c$^+$ cells (63, 65);
5. migration of vast numbers of DCs (presumably Langerhans cells) from site of injection of gp96 to the draining lymph nodes (62);
6. translocation of NFκB into the nuclei of macrophages and DCs, which is perhaps the proximal event that mediates many of the other events listed here (63).

Many of these phenomena have been described for murine and human macrophages and DCs. Two aspects of these studies merit specific discussion. One is

the evidence that contaminating LPS is not responsible for the phenomena observed and that the activity resides in the polypeptide chain of the HSPs. This was demonstrated in several ways including an absence of measurable amounts of LPS in the HSP preparations used in the studies. Other criteria used to demonstrate the LPS-independence of the phenomena were the use of an LPS-antagonist Rslp derived from *Rhodopseudomonas spheroides*, the use of LPS-hypo-responsive mice, lack of requirement of LPS-binding protein (i.e., serum-independence) for the responses, and the differences in kinetics of responses from the kinetics of LPS-induced phenomena. In some studies, the heat or protease sensitivity of the phenomenon has been demonstrated as evidence of lack of involvement of LPS. Not all methods were used in all studies, but more than one method for eliminating LPS as a cause was used in most studies. The second aspect has to do with the quantity of HSPs required for elicitation of responses. Typically, HSP concentrations of 50 μg/ml to 400 μg/ml in vitro were used, although some studies were able to detect the responses with HSP concentrations as low as 1μg/ml. The higher concentrations have been criticized by some reviewers as being unphysiological. Such criticism lacks validity on two counts. First, the molar concentrations at which the HSPs are effective in eliciting the innate responses are not very different from the corresponding concentrations of LPS required to elicit the same responses. Second, the HSPs are the most abundant group of soluble proteins in cells, and if released as a result of cell lysis (see next section), they can achieve extremely high local concentrations. Thus, the APC-activating functions of HSPs can be easily imagined to occur under physiological conditions.

The Unique Position of HSPs at the Intersection of Innate and Adaptive Immune Responses

The above account shows that the mechanisms through which immunization with HSP-peptide complexes elicits its potent anti-tumor, anti-viral, or anti-parasitic effect are now clear. It also illustrates how the interaction of HSPs with peptides and with APCs leads, on one hand, to stimulation of $CD8^+$ and $CD4^+$ T lymphocytes specific for the antigenic peptides chaperoned by the HSPs, and on the other hand, to a cascade of non-antigen-specific events (Figure 3). The latter presumably create the micro-environment necessary for effective functioning of the former. This presumption is supported by our observations (68) that while the tumor-protective activity elicited by immunization with HSP-peptide complexes could be completely abrogated by depletion of $CD4^+$ or $CD8^+$ T cells of the treated mice, it could be abrogated just as completely by depletion of NK cells.

CD91 AND OTHER HSP RECEPTORS ON APCS

The idea of an HSP receptor is, at first sight, counterintuitive. Receptors are meant for detecting molecules that roam the body fluids and other extracellular locations. Hormones are clear examples of the kind of substances for which receptors should exist. HSPs, on the contrary, are quintessential intracellular molecules. They are

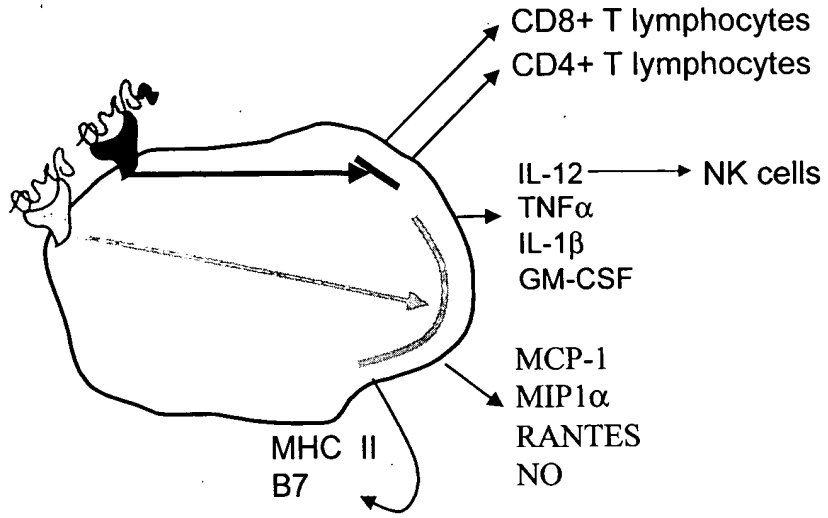

Figure 3 The HSP-APC interaction integrates adaptive and innate immune phenomena. It results in a series of antigen-specific and nonspecific consequences. The antigen-specific consequences (re-presentation of HSP-chaperoned peptides by the MHC I and MHC II molecules and the consequent stimulation of $CD8^+$ and $CD4^+$ T lymphocytes) are mediated through the CD91 HSP receptor (*filled in black*). The antigen-nonspecific consequences (cytokine and chemokine release, DC maturation, etc.) are mediated by other receptors, not yet identified definitively (*filled in gray*).

not found in blood, cerebrospinal fluid, synovial fluid, seminal fluid, or other secretions tested, under normal conditions (P. Srivastava, unpublished data). Why would the body need receptors for such molecules? Let us return to the reasons due to which the existence of HSP receptors was first predicted. HSP receptors were hypothesized simply to explain the mechanism for an artificial phenomenon, the extraordinary immunogenicity of HSP-peptide complexes (54). However, now that one of the HSP receptors has been identified, the physiological logic for its existence (and for existence of other HSP receptors) has come into vivid relief and sharp focus. In this section, I shall discuss the many immunological functions that have been shown to be, and others I predict will be shown to be, mediated through the HSP receptors. The evolutionary logic for the emergence of the HSP receptors also becomes strikingly clear through the studies discussed here and the thoughts that they have inspired.

α2M and the HSPs, A Certain Symmetry (or Alternatively, CD91, the Great Antigen Sampler)

Identification of CD91 as an HSP receptor provides a revealing glimpse into the evolutionary origins of the HSP-APC interaction. CD91 was identified previously as a receptor for the serum protein α2M. α2M is a highly conserved molecule that

can be traced as far back as *Caenorhabditis elegans* and perhaps earlier (70) It is also the evolutionary precursor of the C3 complement component, which in turn is the precursor for C4 and C5. I suggest in the following paragraph that in primitive organisms, α2M is involved in host defense in a manner strikingly analogous to the role of HSPs in host defense in mammals (Figure 4). Invading pathogens elaborate proteases in order to enter the host; it falls upon α2M, the protease inhibitor, to neutralize the proteases and foil the pathogen's designs. Further, the α2M is a protease inhibitor of a most unusual sort; the molecules contain a "bait" region that harbors sequences that are substrates for selected proteases. Once the proteases take the bait, i.e., proteolyse it, the α2M molecule acquires an altered conformation that physically traps the proteases into a molecular basket. The protease, now trapped

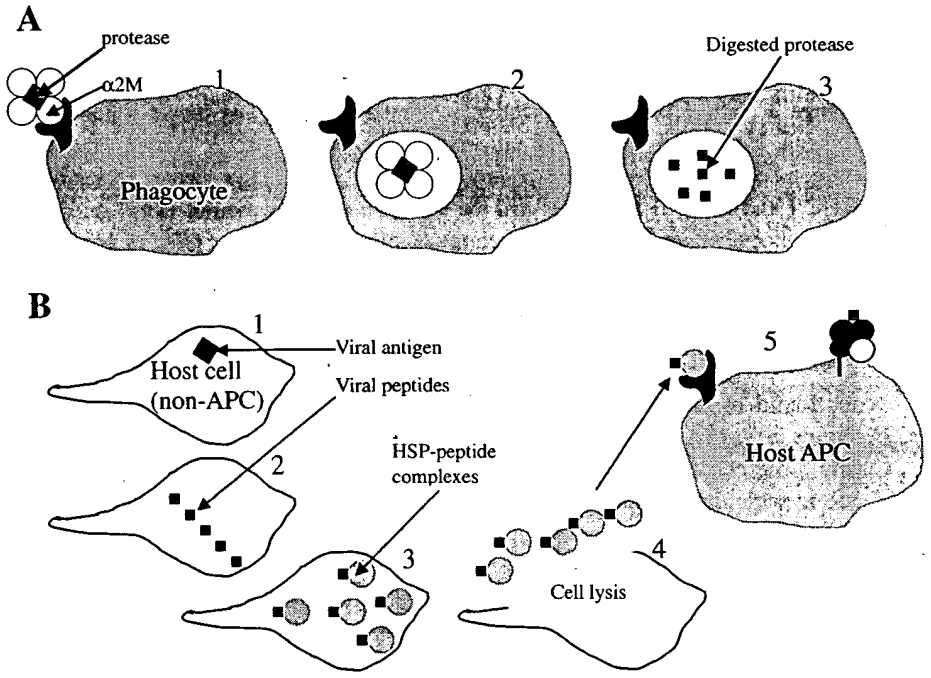

Figure 4 The author's view of how a mechanism of nonspecific surveillance against external pathogens in primitive organisms evolved into an integral part of a complex adaptive immune response to internal pathogens and cancers. Evolution of a mechanism of indirect presentation is inherent in the scheme. (*A*) Inhibition, uptake, and degradation of protease secreted by an *external* pathogen by the host α2M/receptor on phagocytes of primitive organisms. (*B*) Degradation of *internal* pathogen, binding of the peptides by HSPs, lysis of host cell, release of HSP-peptides, their uptake by host α2M/receptor CD91, and re-presentation by MHC I molecules of the vertebrate APC. Those symbols not identified here are the same as in Figures 2 and 3

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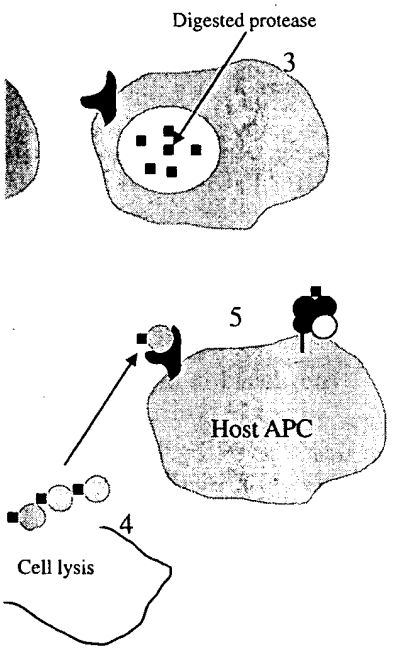

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by α2M, is endocytosed through the α2M receptor CD91 and internalized into the phagocytes, wherein the foreign protease is digested by the host proteases. The digested products, the amino acids, act as a source of nutrition for the host. Thus, α2M combines the functions of host defense and nutrition.

Now, let us fast forward the evolutionary videotape by several hundred million years. The landscape has changed substantially. The pathogens have now acquired sophisticated means of entry into the host other than secreting proteases. Viruses and intracellular parasites have developed ligands for host receptors such that they can enter the host formally through proper channels rather than by breaking down the walls through proteases. Having entered the host cells, they can now establish residence there and replicate. What is the α2M to do? It is a secreted protein selected to patrol the periphery, capture the enemy, and bring it in through the CD91 portal to be digested. It is not trained to scan the intracellular environment. Lo and behold! There has always existed a potent and abundant intracellular agent that happens to come into contact with the entire intracellular contents (including but not limited to invading pathogens) as it helps fold the proteins, chaperones them to interact with other proteins, and helps degrade them when necessary. It is the HSPs. They carry bits and pieces of everything they encounter. They are now co-opted to play their role in defense.

The next steps are simple. The pathogens infect the cells, replicate there, and at some point destroy them. The abundant HSPs carrying the information about the internal environment are released and now dock to the CD91 that has by now developed a far more sophisticated downstream mechanism than simply digesting the enemy (the nutritional needs of the host now being met through an independent agency). The new mechanisms have been selected because the enemy may now look very much like the host; to tell one from the other, a complex and independent adaptive immune system has developed. The CD91 along with the HSPs carrying their information (peptides) is internalized; while some of it still goes to an acidic compartment for digestion of the contents as in the days of yore, the rest of it now goes to a separate nonacidic compartment that channels the peptides through a complex processing pathway that leads to their presentation by the MHC I molecules of the phagocyte (now called the APC). The MHC I molecules simply bind what they can and present them to the T cells, which alone can tell if a given peptide hints at the presence of an enemy. If it does, the T cells expand and go on a search and destroy mission.

This is my personal view of how the surveillance of the external milieu by the α2M/CD91 system came to be transformed into an efficient mechanism of internal surveillance by the HSP/CD91 system (Figure 4). A view of the evolution of the mechanism of indirect presentation is inherent in this opinion. The primordial functions of HSPs as chaperones came to be recruited into the needs of an increasingly complex security apparatus whose challenges had transformed from protecting the host from pathogens that were bluntly violating the host's perimeters to those, i.e., viruses and intracellular parasites, that had learned the ways of the hosts and learned to live within them. The symmetry between α2M and HSPs

becomes evident in this view. This view also makes the CD91 the central antigenic sampling device for both the extracellular and the intracellular antigenic worlds, and this view is further elaborated in a subsequent section where I argue that the HSP receptors, including but not limited to CD91, act as sensors of necrotic cell death and more broadly as sensors of cellular stress.

The symmetry between α2M and HSPs has an interesting practical application. Binder et al. (71) argued that as α2M and HSPs use the same CD91 portal into the APCs and as HSP-peptide complexes entering through this portal are presented through the MHC I molecules, peptides chaperoned by α2M should be similarly processed and presented by MHC I molecules. The α2M-peptide complexes should therefore be effective immunogens as well. This is indeed the case, further demonstrating an additional symmetry between HSPs and α2M.

CD91: Key Portal for Indirect Presentation or Cross-Priming?

CD91 has been shown by Basu et al. to be a common receptor for gp96, hsp90, hsp70, and calreticulin (61). Our ongoing studies indicate that it may indeed be a common receptor for other HSPs as well (R. Binder, P.K. Srivastava, unpublished). Basu et al. make the interesting observation that blocking of CD91 through an antibody to it, or through its ligand α2M, can inhibit completely the phenomenon of re-presentation of peptides chaperoned by any of the HSPs tested. This result indicates not only that CD91 is a receptor for the HSPs tested, but also that it is the sole receptor for them with respect to their peptide re-presenting function. This observation has an important implication for one of the most significant immunological phenomena, indirect presentation or cross-priming. Suto & Srivastava (23) and Arnold et al. (24) have shown previously that HSP preparations can cross-prime, i.e., gp96 preparations from an antigen-positive cell of a given MHC haplotype can immunize mice of a different haplotype and elicit CTL responses restricted by the MHC I of the other haplotype. Our ongoing studies (R. Binder, P.K. Srivastava, unpublished) now indicate that not only gp96, but other HSPs, i.e., hsp70, hsp90, and calreticulin as well, can cross-prime. They further indicate that if HSPs are rendered unavailable, indirect presentation of an antigen cannot occur and that HSPs are necessary for cross-priming or indirect presentation. In other words, in order for an antigen to be re-presented, it must exist in a form that is complexed with one or more HSPs. To the extent that the results of this study are valid and generalizable, it follows that CD91 is a key portal for indirect presentation or cross-priming.

In addition to the experiments discussed above, quantitative considerations propel us into pursuing the idea that HSP-antigen complexes are the preferred form for channeling antigens into the pathway for indirect presentation in vivo. Under physiological conditions, indirect presentation requires tremendous quantitative economy with respect to the antigen. Typically, a small number of cells may be infected by a virus or parasite, and the antigens involved are not necessarily expressed in abundant quantities. Indeed, there is little correlation between the abundance of an antigen and its re-presentability, suggesting that the mechanism that mediates

indirect presentation in vivo is able to do so with very little antigen. This in turn suggests the involvement of receptor-mediated mechanisms. Apart from its biological common sense, this line of reasoning is supported by Lanzavecchia's demonstration (72) that the uptake of antigen for re-presentation by MHC II molecules is more efficient by several orders of magnitude if mediated by a receptor-mediated mechanism (antigen-specific B lymphocytes) than by a receptor-independent mechanism. Of the few mechanisms that have been claimed to be candidates for indirect presentation (12), only two are receptor-mediated, the CD91 HSP receptor-mediated antigen uptake and the uptake of antibody-bound antigens through the Fc receptor (73). Without negating the important role that the latter mechanism must play under certain conditions, HSP-mediated antigen uptake is particularly attractive, as HSPs have been shown to chaperone antigenic peptides from all cellular compartments and of a wide variety, including tumor antigens, viral antigens, minor histocompatibility antigens, and model antigens as discussed earlier. The HSPs thus appear to be a universal mechanism for antigen-capture, and they permit a high-efficiency antigen uptake through a receptor-mediated mechanism.

While much of the excitement about cross-priming has centered around stimulation of $CD8^+$ T cells by MHC I–peptide complexes for obvious reasons, cross-priming also occurs just as readily for MHC II–presented antigens. On the basis of quantitative considerations discussed above, it is my belief that cross-priming for this pathway also occurs through HSP-peptide complexes, although there is no mechanistic necessity for it.

Other HSP Receptors as Key Players in Innate Immunity

The discussion of HSP receptors has focused thus far on the receptor involved in re-presentation of HSP-chaperoned peptides, CD91. This is obviously asymmetrical because the HSP-APC interaction has many significant consequences other than re-presentation of HSP-chaperoned peptides. These include the antigen-independent elaboration of cytokines and chemokines and translocation of NFκB into the nucleus and other effects previously discussed. There is little evidence that CD91 is the receptor involved in these phenomena. Considering that the other phenomena must involve signal transduction, and that it is not clear if CD91 is a signaling receptor, there is a strong likelihood that other receptors are involved. Indeed, there is some evidence for a role for other receptors. Ohashi et al. have implicated the LPS receptor tlr4 in signaling by hsp60 (74), and Panjwani et al. believe CD36 to be a signaling receptor for gp96 (75). These studies are still quite preliminary and have not established that the HSPs actually interact physically with the tlr4 or CD36, nor have these studies established the downstream signaling pathways involved. The lack of maturity of these results notwithstanding, it is reasonable that the identity of these or other receptors, the molecular details of their interactions with HSPs, and the signaling cascades initiated by such interactions will be revealed in the near future. These other putative receptors are most likely to be involved in antigen-independent mechanisms and thus are expected to be the key

players in the innate immune responses initiated by HSP-APC interaction. Since IL-1-like activities are secreted by phagocytes of the most primitive multicellular organisms, the HSP receptor(s) on APCs that mediate secretion of IL-1 are likely to provide the primordial template of a signaling circuit involved in innate immunity.

The discussion in this section has thus far centered on the HSP-APC interaction. Newly emerging evidence indicates that HSPs may interact with cells other than APCs, such as platelets (76), NK cells (77), and T cells (69, 78). These observations are still relatively preliminary and will no doubt reveal novel aspects of the role of HSPs in innate immunity.

HSP Receptors as Sensors of Necrotic Cell Death

The paradox that HSPs are intracellular molecules and yet CD91 and other receptors for them exist has been discussed at the very beginning of this section. The resolution to the paradox lies within it: HSP receptors make perfect sense *because* HSPs are quintessentially intracellular and abundant soluble molecules *under normal conditions*. If extracellular HSPs are detected by a receptor through release of cytokines or chemokines, or maturation and migration in the case of DCs, the conditions must be abnormal. The recent demonstration by Basu et al. (63) that hsp70, hsp90, gp96, and calreticulin are released from cells as a result of necrotic but not apoptotic death is germane in this regard. Nicchitta and colleagues have similarly observed that gp96 is released from cells undergoing virus-induced lytic death but not from cells dying apoptotically (79). Melcher et al. (80) had reported earlier in a pioneering study that tumor cells undergoing necrotic death are highly immunogenic as compared with those undergoing apoptotic death. Necrotic death may not be a physiological event, whereas vast numbers of cells die apoptotically during embryonic develoment, thymic selection, and other processes. Thus, the detection of HSPs by a receptor is an excellent mechanism to signal an abnormal loss of physical integrity. Considering the phylogenetic antiquity of phagocytes and of HSPs, it is safe to suggest that the release of HSPs due to inappropriate necrotic death may be an ancient mechanism for making a host aware that bad things were happening to it. The recent demonstration by Galucci et al. (81) and Sauter et al. (82) that necrotic cell lysates but not apoptotic cells cause maturation of DCs is consistent with these ideas. I believe that HSPs are the major components of the DC-maturing activity of the necrotic lysates, although other components such as DNA also cause activation of APCs (83). These ideas may be generalized to say that necrotic cell death may not be the only event that exposes APCs to HSPs. Stressed cells and cancer cells have been reported to express cell surface HSP molecules (50, 77, 84, 85), and it is tempting to imagine that such cells activate APCs directly. Interestingly, Li and colleagues have observed recently that physical contact of tumor cells artificially engineered to express cell surface HSPs with immature DCs elicits a powerful maturation of DCs (22b). Bhardwaj and colleagues have recently observed that murine and human cancer cells show elevated levels of hsp70 and gp96, and their necrotic lysates have elevated DC-maturing activity (85a). These observations blend seamlessly with the early observation of Menoret et al. (86) and

Melcher et al. (80) who demonstrated that the immunogenicity of tumors co-segregated with expression of inducible but not constitutive hsp70. Vanaja et al. (86a) and Clark & Menoret (87) have shown recently that heat shocked tumor cells that express high levels of inducible HSPs are more immunogenic than their constitutive counterparts. Further exploration of these ideas has now lead Callahan et al. (88) to explore the differences in peptide-binding abilities of inducible versus constitutive hsp70. Whether the inducible HSPs have unique effects on APCs, distinct from the effects of constitutive HSPs, is a fascinating and biologically attractive idea awaiting further experimental attention.

We integrated these ideas and suggested recently that the HSP receptor CD91 is a sensor of necrotic cell death. In view of the recognition that there might be two classes of HSP receptors—CD91 involved in re-presentation and others (possibly tlr4, CD36) involved in the innate components of APC activation—we extend our previous suggestion to say that HSP receptors as a class are sensors of necrotic cell death. This discussion will not be complete without a mention of the elegant work of Fadok et al. (89) who have identified, through an experimental tour de force, a receptor for phosphatidyl serine, a marker for apoptotic cells, on APCs. They further showed that engagement of this receptor activates the anti-inflammatory program in APCs. This phenomenon is the mirror image of our observations that activation of HSP receptors (read, necrosis receptors) activates the pro-inflammatory program in APCs. The interesting possibility must be considered that the HSPs and phosphatidyl serine inversely modulate the APC receptors for the other as a mechanism to maximize their effect on the APCs.

Evolutionary Conservation of HSP-APC Interaction

Following Dobzhansky's dictum that biological phenomena could not be understood unless viewed through the prism of evolution, we sought evidence for immunogenicity of HSP-peptide complexes in the earliest vertebrate that has transplantable tumors and has an adaptive immune system. Inevitably, we arrived at the *Xenopus*. Using a syngeneic transplantable tumor of the *Xenopus*, Robert et al. (90) showed the specific immunoprotective activity of the tumor-derived hsp70 and gp96 against the tumor from which the HSPs were purified. They also showed the dependence of this phenomenon on the presence of HSP-associated peptides. In a cross-cultural experiment, the APCs of mice pulsed with *Xenopus* HSP complexed in vitro with a mouse CTL epitope were able to re-present the *Xenopus* HSP-chaperoned peptide to mouse CTLs. In so doing, we were able to recapitulate in the phylogenetically distant *Xenopus*, the essential elements of HSP-peptide and HSP-APC interaction described thus far in mice, rats, and humans.

HSPS AND MHC: A CIRCLE CLOSED

The common experimental pedigree of the discovery of MHC molecules and of the immunological functions of HSPs was alluded to in the beginning of this chapter. In closing, I would like to comment on the continuing intertwined roles of these

two groups of molecules. The MHC and the HSPs are peptide-binding proteins, although the association is far more promiscuous in the case of one than the other. The differences in the levels of promiscuity have an evolutionary origin and a physiological consequence. The HSPs began to bind peptides at the very beginning of life, as a collateral consequence of their general chaperoning functions. The MHC proteins, on the other hand, were perhaps selected for peptide-binding in an evolutionary era of increasing organismal complexity. The differences in the stringency of requirements for peptide-binding by the two molecules make it possible for one (the HSPs) to take all comers and to render them presentable by the other (the MHC), depending on the latter's more discerning demands. From these differences arises the ability of HSPs (and I believe, an essential function of HSPs) to cross-prime. The innate ability of HSPs to scan the entire protein repertoire of proteins inside the cells, also as a collateral to their normal chaperoning function, allows them to play a key role as informers of the MHC molecules (and through them of the T cells) with respect to any pathogens that might lurk inside the host. The ability of HSPs to interact specifically with the APCs through the HSP receptors, selected I believe as early as the appearance of the phagocyte, imparted on them the ability to be economical with the precious quantities of antigens to be presented to the MHC molecules. Finally, we have long suggested (54) that the HSPs of the cytosol and the ER play a role in chaperoning the peptides from the point of their generation to the point of their being loaded onto the MHC I molecules, and indeed HSPs help process them through their inherent aminopeptidase activity (91). These are some of the many ways in which I believe that the roles of these two molecules are essentially interwoven to form the majestic tapestry of immune response. We took these ideas far, perhaps too far, and suggested in 1991 that the lack of any obvious sequence homologies between the MHC and the HSPs notwithstanding, the two molecules may have common evolutionary precursors. The emerging structural evidence with respect to hsp70 does not support that notion (35), although the gp96 molecule has recently been modeled on the basis of the MHC I molecule (38). Regardless of what the structures tell us in the future, the functional convergence of the MHC and the HSPs is increasingly apparent and esthetically appealing; esthetic appeal remains an undeniably excellent basis on which to form experimentally testable hypotheses.

This is an appropriate place to comment on some of the experimental avenues that have been so productive in exploration of structure and function of the MHC and that are not available for the study of the HSPs. The lack of polymorphisms and of naturally arising HSP mutants, the consequent inability to do genetics, and to extinguish expression of HSPs in cells in culture or in vivo due to cellular or embryonic lethality are some of the obvious experimental limitations that result from the vital roles of the HSPs in nonimmunological processes. The lack of these tools has made it difficult to pin down many of the roles of HSPs in immune response with the finality that one would like. Evidence for many of the phenomena described here therefore was circumstantial rather than direct, the more so when the observations or the interpretations were first made. Nonetheless, the observations

have firmly stood the test of independent reproducibility, and the interpretations and predictions have, without exception, proven to be correct. Altogether, the evidence for a fundamental physiological role of HSPs in an array of immunological phenomena continues to widen.

THE ROADS NOT TAKEN

This area of work began with exploration of the biochemical basis of immunogenicity of tumors (39). It would have been appropriate to end it with a discussion of the clinical applications of the ideas that have come out of this exploration. However, space does not permit that description, nor does it permit a discussion of the applications of the HSP approach for immunotherapy of infectious diseases. The reader is referred to other reviews for these purposes (45, 92). Space also does not permit discussion of the dose-restriction of immunogenicity of HSP-peptide complexes and its implications for generating downregulatory antigen-specific T cells (93, 94). Finally, the idea that HSPs chaperone antigenic peptides from the point of their generation in the cytosol to their being loaded onto MHC I molecules in the ER, proposed some time ago (54), and an idea that has been as hard to prove as it has been to disprove, but which continues to gather support (91, 95–97a), is not discussed here. These omissions are solely for reasons of limitations of space.

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in vivo *14:* 571-585 (2000)

Cancer Vaccines: An Update

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Abstract. *This review summarizes the most recent findings and the future directions in designing cancer vaccines. The newest tumor-associated antigens and the most promising approaches to cancer vaccine development are discussed. We categorized them as follows: peptide vaccines, recombinant viral vaccines, DNA vaccines, dendritic cell-based immunotherapy, and the use of heat shock proteins and adjuvants. We focus on their advantages and disadvantages in addition to clinical potential.*

The possibility for the development of cancer vaccines was first recognized in 1893 by the New York surgeon William Coley who reported the regression of several human sarcomas following immune stimulation with a bacterial toxin. Renewed interest in cancer vaccines today is based on two recent advances which have allowed the design of more specific vaccine approaches: improved molecular techniques for the identification of genes encoding tumor-associated antigens, and better understanding of the mechanisms involved in antigen processing, presentation, and T cell activation. T cells expressing CD4 molecules recognize peptides of 12-25 amino acids presented by MHC class II molecules (1). The cytotoxic T-lymphocytes (CTL) expressing CD8 molecules recognize class I restricted peptides of 8-10 residues which are the products of intracellularly processed proteins (2). Cytosolic peptides are transported across the endoplasmic reticulum (ER) membrane with the help of the ATP-dependent transporters associated with antigen processing (TAP) (3). Peptides complexed with class I molecules in the ER are then transported to the cell surface for recognition by CTL (2). The interaction between CTL and the target tumor cells begins with the binding of the peptide antigen associated with MHC class I molecule to the T cell antigen receptor. Lymphocyte-mediated cytolysis is further enhanced by accessory molecules such as lymphocyte function antigens (LFA-1 and LFA-3), co-stimulatory molecules (CD28, B7), and intercellular adhesion molecule (ICAM-1) (4).

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Key Words: Cancer vaccines, tumor-associated antigens, antigen presentation, synthetic peptides, signal sequence, recombinant vaccines, immunotherapy.

Identification of tumor-associated antigens

The identification of several tumor-associated antigens (TAA) in melanoma and other cancers has been an important development in the field of tumor immunology. A variety of approaches have been used for the identification of TAA recognized by CTL. Most of the melanoma antigens have been identified by screening cDNA expression libraries with CTL reactive against melanoma (5).

Another approach for the identification of TAA involves testing of known proteins for recognition by CTL. With this approach, Kawakami *et al.* found that the expression of tyrosinase and gp100 correlated with lysis by HLA-A2-restricted, melanoma-reactive CTL (6). The same investigators demonstrated later that HLA-A2+ cell lines transfected with the gene encoding gp100 can be recognized by melanoma-reactive CTL (7). Tyrosinase gene product was also recognized by HLA-A2-restricted CTL (8).

Direct isolation and sequencing of peptides eluted from the tumor cells is another method of identifying tumor-associated peptide antigens. Several groups have used this approach to isolate peptides recognized by melanoma-specific CTL (9), as well as to sequence the peptides with a triple quadriple mass spectrometer (10). This technique is complementary to the genetic approach because it allows measurement of the abundance of the antigenic peptides derived from the gene sequence. This is very important for the recognition of tumor cells by CTL, because at least 200 molecules of a peptide must occupy MHC class I molecules in order for CTL to lyse cancer cells (11). Another advantage of this technique is the direct identification of peptides naturally processed and presented on the tumor cell surface.

More recently, computer programs have been used to identify peptide sequences of known proteins based on their binding affinity for selected HLA molecules (12). We analyzed the sequence of human telomerase reverse transcriptase (hTRT) (13) for peptide sequences containing known binding motifs for the HLA-A2.1 molecule (14). We

0258-851X/2000 $2.00+.40

also used the software of the Bioinformatics & Molecular Analysis Section (National Institutes of Health, Washington, DC) available at http://bimas.dcrt.nih. gov/molbio/hla_bind/index.html which ranks 8-10 mer peptides based on a prediction half-time dissociation coefficient from HLA class I molecules (15). We tested whether two of the highest-ranking peptides can generate *in vitro* CTL able to recognize peptide-pulsed targets and HLA-A2+ cancer cells. We demonstrated in this study that the hTRT-specific CTL of normal individuals and patients with prostate cancer specifically lysed a variety of HLA-A2+ cancer cell lines, suggesting the existence of precursor CTL for hTRT in both normal individuals and in cancer patients (16). Since telomerase activity is increased in the vast majority of human tumors, our findings could contribute to the generation of universal telomerase-based cancer vaccines.

Serological analysis of recombinant cDNA expression library of human tumors with autologous serum (SEREX) is another approach used to isolate human tumor antigens (17). Examples are tyrosinase, MAGE, NY-ESO-1, SSX2, SCB-1, and CT7 (5). Some of these antigens are T-cell defined antigens, which emphasizes the usefulness of SEREX analysis in identifying new tumor antigens.

Melanoma antigens. Human melanoma antigens can be classified into three groups: (i) antigens expressed in melanoma, normal melanocytes, and retina; (ii) antigens expressed in several cancers and testis; and (iii) antigens specific for individual tumors.

The first group consists of nonmutated shared tumor antigens. An interesting correlation between depigmentation of skin and hair and good clinical responses to chemotherapy and immunotherapy (18), suggests that the same population of CTL recognizes both melanoma antigens and nonmutated shared antigens on melanocytes. Rosenberg *et al.* observed tumor regression in patients who developed vitiligo after interleukin (IL-2)-related immunotherapy, suggesting that autoreactive CTL may be involved in tumor regression (19). Tyrosinase, MART-1/Melan-A, gp100, TRP1/gp75, and TRP2 have been identified as shared melanoma antigens recognized by CTL (5). These antigens may form the basis for the development of effective vaccines, but their expression on normal tissues raises concerns about the possible development of immunological tolerance and autoimmunity associated with the immunotherapy.

The second group includes several families of antigens, specifically: MAGE, BAGE, GAGE, RAGE, and NY-ESO-1. The MAGE genes are silent in a large panel of healthy adult tissues, with the exception of testis and placenta (20). Recently, 5 MAGE-A1 epitopes recognized by CTL were identified by *in vitro* stimulation with dendritic cells transduced with a recombinant canarypoxvirus (ALVAC) containing the entire MAGE-A1 gene (21). Like the MAGE genes, BAGE (22) and GAGE (23) genes are predominantly expressed in melanomas. Another gene called RAGE (renal carcinoma antigen gene) (24) is also expressed in melanomas, sarcomas and bladder tumors. Another antigen in this group is NY-ESO-1. It is not expressed in normal human tissues except testis but is frequently expressed in melanoma, breast, prostate, bladder, lung carcinoma, and other types of cancers (25). Interestingly, both HLA-A2 and HLA-A31 restricted T-cell epitopes have been identified from its primary open reading frame (26). More recently, Jeager *et al.* identified 3 NY-ESO-1 epitopes presented by HLA class II molecules and recognized by CD4+ T lymphocytes of 2 melanoma patients (27). Since these antigens are expressed in a variety of cancers but not in healthy tissues, they may be appropriate targets for immunotherapy.

Finally, some antigens unique to individual tumors appear through tumor-specific mutations, deletions or recombination events. A point mutation might change a normal peptide unable to bind to MHC molecules into a peptide capable of binding to MHC and, therefore, presented to the immune system. Natural tolerance eliminates any CTL recognizing normal peptides capable of binding to MHC. In case of a point mutation however, the modified peptide may become a target detected by existing CTL. Several antigens generated by point mutations on a murine tumor were recognized by autologous CTL (28). Point mutations were also found to encode human tumor antigens recognized by CTL (29) (30) (31). This group of antigens should be recognized by melanoma-specific CTL because their precursors should not have been depleted by the process of natural self-tolerance. From the clinical perspective, however, these antigens may not be useful for development of cancer vaccines because of their restriction to very few individual tumors.

Other tumor-associated antigens. In breast cancer and other adenocarcinomas, a polymorphic epithelial mucin (PEM) has been characterized as a tumor antigen (32) (33) (34) (35). Mucins are high molecular weight glycoproteins. The MUC-1 mucin consists of a heavily glycosylated tandemly repeating 20-amino acid sequence, specifically PDTRPAPGSTAPP-AHGVTSA (32). Aberrant glycosylation of mucins on carcinomatous epithelial cells leads to the exposure of novel core epitopes that are recognized by cytotoxic T cells (33). Even though HLA-unrestricted recognition of MUC-1 has been reported (33) (34), the establishment of mucin-specific cytotoxic T cell lines (34) (35) was a very important achievement in the attempts to develop cancer vaccines targeting this antigen. More recently, MHC-restricted CTL epitopes from non-variable number of tandem repeat sequence of MUC-1 have been identified (36). Since PEM is much more highly expressed on carcinomas than on normal tissues, it could be a suitable target for immunotherapy.

The HER2/neu protooncogene, expressed in breast cancer and other human cancers, encodes a tyrosine kinase with homology to epidermal growth factor receptor, with a relative molecular mass of 185 kd (37). HER2/neu protein is a receptor-like transmembrane protein comprising a large

cysteine-rich extracellular domain that functions in ligand binding, a short transmembrane domain, and a small cytoplasmic domain (37). HER2/neu is amplified and expressed in many human cancers, largely adenocarcinomas of breast, ovary, colon, and lung. In breast cancer, HER2/neu overexpression is associated with aggressive disease and is an independent predictor of poor prognosis (38). Several class I restricted HER2/neu-derived peptides which were recognized by breast and ovarian cancer-specific cytotoxic T lymphocytes have been described (39) (40) (41).

In contrast to class I TAA, little attention has been paid to the identification of class II TAA, mostly because of the difficulties in their identification. However, a growing number of studies confirm the important role of CD4+ T cells in controlling tumor growth (42). In addition to tyrosinase (43), MAGE-3 was also recognized by CD4+ T cells which were generated by *in vitro* stimulation of peripheral blood mononuclear cells (PBMC) with dendritic cells (DC) pulsed with synthetic peptides or purified MAGE-3 protein (44). One novel genetic approach was recently developed for cloning genes encoding MHC class II restricted tumor antigens. This approach allows for the screening of an invariant chain-cDNA fusion library in a genetically engineered cell line expressing the essential components of the MHC class II processing pathways. The first antigen identified with this approach was CDC27, which is recognized by CD4+ HLA-DR4-restricted tumor infiltrating lymphocytes (45). It was recently reported that a MART-1-derived peptide presented by HLA-DR4 was able to induce the *in vitro* expansion of specific CD4+ T cells derived from normal DR4+ donors or from DR4+ patients with melanoma when pulsed onto autologous DC (46). This study found that CD4+ T cell immunoreactivity against this peptide coexisted with a high frequency of anti-MART-1_{27-35}-reactive CD8+ T cells in freshly isolated blood harvested from HLA-A2+/DR4+ patients with melanoma. Another recent study with cancer patients demonstrated the essential role of DC that are activated by CD4+ Th cells for optimal CTL induction (47). These findings confirm that tumor-specific CD4+ T lymphocytes from cancer patients are required for optimal induction of CTL against the autologous tumors. Therefore, both class I and class II peptides could be used to optimize the therapeutic effect of the immunotherapy for melanoma.

Peptide vaccines

The identification of peptide sequences recognized by CTL has led to attempts to directly induce CTL-responses *in vivo* (48) (49). We showed in two murine antigenic systems that fusion peptides with an synthetic ER-signal sequence at the NH2-terminus of the minimal peptide were more effective than the minimal peptide alone in generating specific CTL-responses (50). Furthermore, we found that the CTL response was MHC class II independent, could not be attributed to increased hydrophobicity of the fusion peptides, and was very effective in prolonging the survival of tumor-challenged mice.

Increasing number of studies report peptide vaccination of cancer patients. Immunizations with a MAGE-3-derived peptide without any adjuvant induced limited tumor regressions in five out of 17 patients with melanoma (51). Salgaller *et al.* reported generation of CTL specific for one of three gp100-derived peptides in patients vaccinated with peptide in incomplete Freund's adjuvant (52). Immunization of three patients with advanced melanoma with peptide-pulsed autologous antigen presenting cells led to induction of peptide specific CTL (53). The peptide used in this study was derived from MAGE-1 and was restricted to HLA-A1.1. The lack of any therapeutic response observed in this trial might be explained by the advanced stage of the disease in these patients. In another study nine melanoma patients were vaccinated weekly for four weeks with a combination of peptides derived from the MART-1, tyrosinase, and gp100 proteins (54). Successful immunization against peptides could be detected *in vitro* in two of six patients against the tyrosinase peptide, three of six patients against the MART-1 peptide, and none of six patients receiving the gp100 peptide. More recently, eighteen patients with melanoma were immunized with a peptide derived from MART-1, emulsified with incomplete Freund's adjuvant (49). An enhancement of cytotoxic activity against MART-1 was detected with minimal toxicity for the patients consisting of local irritation at the site of vaccination. Serial administrations of this peptide appeared to boost the level of cytotoxicity *in vitro*, although clinical regression of the tumor was not observed. In another trial, patients with advanced pancreatic carcinoma were vaccinated with a synthetic ras peptide pulsed on antigen presenting cells isolated from peripheral blood (55). This procedure led to generation of cancer cell-specific cellular response, without side effects. However, in all patients tumor progression was observed after the vaccination.

Several strategies for modifying peptides have been attempted to improve their efficiency as cancer vaccines. The clinical use of peptides is limited by their rapid proteolytic digestion. To overcome this limitation Celis *et al.* designed a peptide construct containing a pan-reactive DR epitope, a CTL epitope and a fatty-acid moiety (56). A lipopeptide-based therapeutic vaccine was able to induce strong CTL responses both in humans and animals (57). Several studies demonstrated a correlation between MHC binding affinity and peptide immunogenicity (58). Peptides derived from gp100, whose anchor residues were modified to fit the optimal HLA-A2 binding motif, stimulated tumor-reactive CTL more efficiently than the natural epitopes (59). An unmodified, gp100-derived peptide, failed to elicit peptide-specific CTL in melanoma patients after subcutaneous administration with incomplete Freund's adjuvant (IFA). In contrast, vaccination with the modified peptide induced CTL responses in 91% of cases (60). None of the 11 patients

immunized with the modified peptide in IFA alone experienced an objective tumor response. Interestingly, administration of the modified peptide along with high dose interleukin-2 led to a clinical response rate of 42% in a group of 31 patients. Several groups reported clinical trials with melanoma patients immunized with the immunogenic peptide MART-1_{27-35} (AAGIGILTV) (61) (62) (63). Wang *et al.* immunized patients with high-risk resected melanoma with MART-1_{27-35} complexed with incomplete Freund's adjuvants, or with Freund's adjuvants mixed with CRL1005, a blocked co-polymer adjuvant. Ten of 22 patients demonstrated an immune response to peptide-pulsed targets or tumor cells by ELISA assay after vaccination, as did 12 of 20 patients by ELISPOT. Immune response by ELISA correlated with prolonged relapse-free survival (61). This data suggests that a significant proportion of patients with resected melanoma mount an antigen-specific immune response against MART-1_{27-35}. Another more recent study analyzed antigen-specific T-cell responses induced in the skin and in peripheral blood lymphocytes in a HLA-A2+ melanoma patient. The patient showed major regression of metastatic melanoma under continued immunization with peptides derived from the antigens MART-1, tyrosinase and gp100 (62). The authors demonstrated that intradermal (i.d.) immunization with peptides alone leads to oligoclonal expansion of MART-1-specific CTL. These findings provide strong evidence for the effective induction of specific T-cell responses to MART-1 by i.d. immunization with peptide alone, which accounts for specific cytotoxicity against MART-1-expressing melanoma cells and clinical tumor regression. Brinckerhoff *et al.* evaluated the stability of the same peptide - MART-1_{27-35} in fresh normal human plasma and possible peptide modifications that convey protection against enzymatic destruction without loss of immunogenicity (63). When this peptide was incubated in plasma prior to pulsing on target cells, CTL reactivity was lost within 3 hours. The stability of MART-1_{27-35} was markedly prolonged by C-terminal amidation and/or N-terminal acetylation, or by polyethylene-glycol modification of the C-terminus. These modified peptides were recognized by CTL. This study suggests that the immunogenicity of the peptide vaccines might be enhanced by creating modifications that increase their stability.

HER-2/neu-derived peptides were also evaluated for their ability to bind to MHC class I molecules *in vitro* (40). It was found that amino acid substitutions at positions 1 and 9 could improve the HLA binding of these peptides without interfering with recognition by HER-2/neu-specific CTL. An interesting strategy was used to enhance the immunogenicity of a peptide derived from human carcinoembryonal antigen (CEA) (64). Four amino acid residues in this peptide, predicted to interact with the T cell receptor (TCR), were replaced. This change led to more efficient sensitization of CTL, yet unassociated with improved binding to HLA-A2 molecules. These CTL were shown to recognize both the original and the modified peptide, as well as human tumor cells expressing CEA. Therefore, amino acid modifications optimizing the binding of the peptides to MHC molecules, or their interactions with the TCR, might be useful in designing peptide vaccines for cancer. More recently, patients with breast and ovarian cancer were immunized with groups of peptides derived from the HER-2/neu extracellular domain or intracellular domain mixed with GM-CSF as an adjuvant (65). All of the patients immunized with HER-2/neu peptides developed HER-2/neu peptide-specific T-cell responses. Immune T-cells elicited by vaccination were shown to migrate outside the peripheral circulation by virtue of generating delayed type hypersensitivity (DTH) responses distant from the vaccine site, which indicated the potential ability to traffic to the site of tumor.

Prostate-specific antigen (PSA)-based vaccine was able to induce PSA-reactive effector cells after vaccination of patients with prostate cancer (66). The vaccine consisted of recombinant PSA with lipid A formulated in liposomes. This vaccination induced T-cell responses in 8 of 10 patients with prostate cancer. The observed cellular responses were predominantly mediated by CD4+ T lymphocytes.

Twelve patients with chronic myelogenous leukemia were vaccinated with increasing doses of bcr-abl oncogene breakpoint fusion peptides mixed with QS-21 as an adjuvant (67). In 3 of the 6 patients treated at the 2 highest dose levels of vaccines, peptide specific proliferative responses were generated that lasted up to 5 months after vaccination. In this study, CTL responses have not been observed.

We investigated the effectiveness of several synthetic insertion signal sequences in enhancing the presentation of the HLA-A2.1 restricted melanoma epitope MART-1_{27-35} (68). An important step in presentation of the class I-restricted antigens is the translocation of processed proteins from the cytosol across the endoplasmic reticulum membrane mediated by transporter associated with antigen processing proteins (TAP), or as an alternative, by endoplasmic reticulum-insertion signal sequences located at the NH2-terminus of the precursor molecules (69). Using a technique known as osmotic lysis of pinocytic vesicles, we loaded several synthetic peptide constructs into the cytosol of antigen processing deficient T2 cells, TAP-expressing human melanoma cells, and dendritic cells (70). We examined whether the natural signal sequences ES (derived from the adenovirus E3/19K glycoprotein) (71) , and IS (derived from IFN-ß) (72) could enhance and prolong presentation of MART-1_{27-35}. We found that the addition of signal sequence at the N-terminus, but not at the C-terminus, of MART-1_{27-35} greatly enhanced its presentation in both TAP-deficient and TAP-expressing cells. A newly designed peptide construct, composed of the epitope replacing the hydrophobic part of a natural signal sequence, was also effective. Interestingly, an artificial signal sequence containing the epitope was the most efficient construct for enhancing its presentation. These peptide constructs facilitated epitope presentation in a TAP-

independent manner when loaded into the cytosol of TAP-deficient T2 cells. In addition, loading of these constructs into TAP-expressing melanoma cells also led to a more efficient presentation than the loading of the minimal peptide. Most importantly, loading of human dendritic cells with the same constructs resulted in a prolonged presentation of this melanoma epitope (73) . The efficient presentation of MART-1_{27-35}, loaded into TAP-expressing tumor cells and DC, may be explained by the availability of intact TAP transporters in these cells. In this case, some of the loaded MART-1_{27-35} may have been translocated by TAP from the cytosol even 8 days after loading. The size of MART-1_{27-35} (9 amino acids) is appropriate for optimal translocation by TAP (3) . Still, fusion peptides were more effective than MART-1_{27-35}, probably because of their translocation by both TAP-dependent and TAP-independent pathway. The later mechanism of peptide translocation may be important for antigen presentation especially in cancers that fail to utilize the classical MHC class I pathway (74) . These findings may be of practical significance for the development of synthetic anticancer vaccines and *in vitro* immunization of CTL for adoptive immunotherapy.

From a clinical perspective, immunization with peptides may be preferable to immunization with recombinant vaccinia viruses because of its safety and because it is not associated with diminished immune responses in patients immunized against smallpox. Immunizing with minimal determinant constructs may avoid the possible oncogenic effect of full length proteins containing ras, p53 or other potential oncogenes. In addition to their safety, peptide vaccines can be designed to induce well-defined immune responses, and synthesized in large quantities with very high purity and reproducibility. Another potential advantage of peptide vaccines over whole proteins or DNA vaccines, is the ability to identify the specific epitopes of the tumor antigens to which an individual is able to mount an immune response, but not a state of immune tolerance (75) . In addition, *in vivo* or *in vitro* immunization with peptide antigens "packaged" in DC or other antigen-presenting cells (discussed below) opens an exciting opportunity for eliciting powerful CTL-responses.

A disadvantage of peptide vaccines is their poor immunogenicity and monospecificity of the induced immune response. Another limiting factor for the use of peptide vaccines in outbred populations is that T cells from individuals expressing different MHC molecules recognize different peptides from tumor or viral antigens in the context of self MHC. However, the use of synthetic peptides from tumor associated antigens that are presented by common MHC molecules may overcome this problem. Poor immunogenicity caused by rapid degradation of the peptides by serum peptidases may be corrected by modifications or incorporation of the peptides into controlled release formulations.

Recombinant viruses

Many different viruses have been used to construct recombinant vaccines. These vaccines have the advantage of inducing both humoral and cell-mediated immune responses, in some cases even after a single application. However, possible disadvantages of recombinant viruses include recombinantion with wild type viruses, conversion to virulence, oncogenic potential, or immunosuppression. We will briefly discuss current strategies to overcome some of these obstacles in order to develop efficient recombinant viral vaccines.

Vaccinia virus (VV) was demonstrated to be a safe and very effective immunogen in the smallpox eradication campaign, where it was administered to over one billion people. Large amounts of foreign DNA can be stably inserted into the VV genome by homologous recombination (76). Another advantage of this vector is a very efficient post-translational processing of the inserted genes within host cell cytoplasm. However, due to the induction of high titers of anti-vaccinia antibodies, recombinant vaccinia viruses may be given only once or twice (77). It was demonstrated that intratumoral inoculation of vaccinia virus induced very high levels of antivaccinia antibodies in serum. Surprisingly however, it was possible to sustain viral gene function by repeatedly injecting vaccinia in the tumor site (78). A promising new strategy to increase the efficiency of recombinant viral vaccines is to use two different vectors for priming and boosting vaccinations (79). This approach was much more effective in generating antigen-specific CTL responses than the use of one vector for both priming and boosting.

Since vaccinia is a replication competent virus, it may cause disseminated viremia especially in immunosuppressed individuals (80). Therefore, several research groups attempted to develop recombinant vaccines based on non-replicating viruses (81). Utilizing recombinant fowlpox virus, which does not replicate in mammalian cells, Wang *et al.* were able to treat established tumors in mice (82). An important aspect of this work was the finding that prior immunization with vaccinia virus did not abrogate the immune responses elicited by the recombinant fowlpox virus. A different non-replicating virus, canarypox virus (ALVAC), was used to generate recombinant viruses, able to elicit immune responses against a variety of antigens (83). A clinical trial with vaccinia-CEA in patients with colorectal cancer resulted in eliciting of cell-mediated immune responses against CEA-derived peptide (84). In this study rejection of the vaccinia virus itself was not observed. More recently, the same group performed the first clinical trial with a nonreplicating ALVAC-CEA vector in patients with advanced carcinoma (85). Although no objective antitumor responses were observed, the vaccine was very well tolerated and no significant toxicity was reported. In 7 of 9 patients evaulated, statistically significant increases in CTL precursors specific for CEA were observed in PBMC

after vaccination. T cell responses elicited by patients before and after vaccination with the ALVAC-CEA recombinants were further characterized in another study (86). This study demonstrated the ability to vaccinate cancer patients with an avipox recombinant as well as to derive T cells that are capable of lysing allogenic and autologous tumor cells in a MHC-restricted manner. Phase I trial of a recombinant vaccinia virus encoding CEA in 20 patients with metastatic adenocarcinoma showed that the toxicity was limited to local inflamation as well as low grade fever, each affecting fewer than 20% of the patients (87). No objective clinical responses to the vaccine were observed among this population of patients with widely metastatic andenocarcinoma. The antibody response to CEA in patients was studied by Conry *et al.* (88). This group used recombinant vaccinia viruses encoding full-length of internally deleted cDNAs for human CEA to vaccinate 32 patients with CEA-expressing adenocarcinomas of colorectal origin. The detected CEA autoantibodies were predominantly IgG1, with a minority of patients also demonstrating IgM autoantibodies. A non-replicating vaccinia virus, known as modified vaccinia virus Ankara (MVA), is avirulent in normal and immunosuppressed animals and was shown to have no significant side effects after inoculation of 120,000 humans (89). Since replication of MVA is blocked at a step of virion assembly (90), rather than at an early stage, MVA vectors produce recombinant proteins in amounts similar to those of wild type viruses. In addition, the immunogenicity of MVA recombinants in mice is similar to that of virulent strains (91). Therefore, MVA is a very promising vector for the development of recombinant vaccines for cancer. This vector was recently used for expression of human tyrosinase, a melanoma specific differentiation antigen (92). Stable recombinant viruses (MVA-hTyr) were constructed that have deleted selection marker lacZ and efficiently expressed human tyrosinase in primary human cells and cell lines. An efficient tyrosinase- and melanoma-specific CTL response was induced *in vitro* using MVA-hTyr-infected autologous dendritic cells as activators for PBMC derived from HLA-A2.1-positive melanoma patients, despite prior vaccination against smallpox. A new recombinant poxvirus vaccine that codes for 10 HLA-A2-restricted epitopes derived from 5 melanoma antigens conjoined in an artificial polyepitope or polytope construct was recently designed (93). Multiple epitopes within the polytope construct were shown to be individually immunogenic, which illustrated the feasibility of the polytope approach for melanoma immunotherapy. Tumor escape from CTL surveillance, through down regulation of individual tumor antigens and MHC alleles, might be overcome by polytope vaccines, which simultaneously target multiple cancer antigens. Fifty-four patients with metastatic melanoma were immunized with recombinant adenoviruses encoding MART-1 and gp100 melanoma antigens alone, or followed by the administration of IL-2 (94). One of 16 patients receiving the recombinant adenovirus MART-1 alone, experienced a complete clinical response. However, immunologic assays showed no consistent immunization to the MART-1 or gp100 transgenes expressed by the recombinant adenoviruses. This study found that high doses of recombinant adenoviruses could be safely administered to cancer patients. Another study tested a recombinant adeno-associated virus expressing human papillomavirus type 16 E7 peptide DNA fused with heat shock protein DNA as a potential vaccine for cervical cancer (95). It was demonstrated that this vaccine can eliminate tumor cells in syngeneic animals and induce CD4- and CD8-dependant CTL activity *in vitro*. This study indicates that this chimeric gene delivered by adeno-associated virus has potential as a cervical cancer vaccine. Prostate cancer recurrence, evidenced by rising PSA levels after radical prostatectomy, is an increasingly prevalent clinical problem. A clinical study was undertaken to evaluate the safety and biologic effects of vaccinia-PSA (PROSTVAC) administered to 6 patients with post-prostatectomy recurrence of prostate cancer (96). Toxicity was minimal, and primary anti-PSA IgG antibody activity was induced after vaccinia-PSA immunization in one subject, although such antibodies were detectable in several subjects at baseline.

These early clinical studies with recombinant viruses as vaccine vectors are very encouraging. In contrast to other vaccine vectors, viruses elicit strong and long-lasting immune response, and are able to infect nearly all host cells, as well as to ensure intracellular translation, degradation and efficient trafficking of peptide antigens to the cell surface. The potential drawbacks of the viral vectors are related to their safety and pre-existing immunity, particularly to vaccinia virus and adenoviruses. However, the safety of the viral vaccines can be ensured by using non-replicating, highly attenuated or genetically modified viruses, while the problem of pre-existing immunity may be circumvented by the use of non-mammalian viruses, such as the avian poxviruses. Therefore, the use of recombinant viruses as cancer vaccines is very promising.

DNA vaccines

This approach involves direct inoculation of expression plasmids, which results in the induction of long-lasting immune responses against the expressed antigens. Fynan *et al.* compared six routes of inoculation of naked DNA for their relative efficiencies (97). In this study, intramuscular injection of DNA generated the best response, whereas inoculation of DNA-coated gold particles using "gene gun" required significantly lower doses of DNA. It was found that the uptake of the injected DNA is an active energy-dependent process (98). Once inside the cell, plasmid DNA can get through the nuclear membrane and persists as a non-replicating episomal molecule, which explains the long-lived foreign gene expression (99). The low, but long-lasting expression of the encoded antigens is an important feature of this approach (100). The duration of expression seems to be more important than the dose of the antigen for induction of

CTL responses, although DNA immunization has been shown to result in both cellular and humoral immune responses, and in generation of antigen-specific CD8+ and CD4+ T cells (101). Several elegantly designed studies addressed the important question of the mechanism of DNA immunization (102) (103). Results demonstrate that the antigen presenting cells (APC) can be transfected directly or they can acquire the antigens expressed by other transfected cells. However, only professional APC are able to initiate primary immune responses as a result of DNA immunization. These findings are extremely important in the development of DNA-based vaccines for clinical application.

Transduction of tumor cells with genes encoding immunologically active molecules created a whole new generation of cancer vaccines (104). Most of the clinical trials evalutating genetically modified tumor vaccines used a variety of cytokine genes: GM-CSF (105), IL-2 (106), IL-4 (107), IL-7 (108), interferon-g (109), and IL-12 (110). Because of the significant variability of the transduced genes, small number of patients involved, and many other variable parameters, statistically significant evaluation of these early clinical trails is impossible at this time.

DNA vaccines have several potential advantages over peptide and recombinant viral vaccines. DNA vaccines are simpler and cheaper to produce. DNA immunization is not associated with an anamnestic immune response, which is responsible for the rapid clearance of viral constructs. Another major advantage is that DNA vaccination induces very long-lasting immune responses. Addressing a major concern for the clinical use of DNA-based vaccines, their safety, Kurth *et al.* calculated that the probability of tumor-promoting events by plasmid DNA integration was below the statistical events leading to a mutation within the lifetime of an individual (111). In all, DNA-based vaccines seem to be a promising approach for the treatment of cancer.

Dendritic cells

A growing number of studies report the successful use of dendritic cells (DC) for inducing anti-tumor immune responses in both animals and patients. DC are the most potent antigen-presenting cells for the initiation of antigen-specific immune responses (112). In addition to their ability to efficiently acquire and process antigens (113), DC express high levels of MHC class I and class II molecules as well as costimulatory molecules (114), essential in antigen presentation. Therefore, many investigators attempted to immunize with peptide-pulsed DC. It was found that immunization with peptide-pulsed DC is superior to injection of peptide in adjuvant in inducing potent cytotoxic T-cell responses (115). A possible disadvantage of peptide pulsing is the short half-life (2-10 hours) of most MHC-restricted epitopes (116), which creates the requirement for several injections of peptide-pulsed DC to achieve effective immune responses (117). Therefore, development of different methods for loading of antigens allowing DC to utilize their own intracellular pathways is highly desirable. The antigens of interest must be present in the cytosol of the DC in order to enter the intracellular pathway, leading to their loading onto MHC class I molecules and the subsequent activation of CD8+ T cells. It was shown that treatment of pulmonary metastases in mice with bone marrow-derived DC transduced with retroviral vector encoding a model antigen was very effective (118). The reduction of the metastatic nodules was associated with induction of antigen-specific CTL. Adenovirus vectors were also used to transduce DC with genes coding for tumor antigens. It was demonstrated in a murine breast cancer model that a single injection with transduced DC provided complete protection against tumor cell challenge (119). This approach was not limited by hepatic toxicity and the development of neutralizing antibodies associated with the direct administration of the adenoviral vectors (120). Another study also suggested that adenovirus vectors are a promising vehicle for genetically engineering of human DC (121). A comparison of various gene transfer methods in human DC showed that adenovirus vectors were the most efficient in transducing human DC, with transduction efficiencies exceeding 95% at higher multiplicity of infection. With our signal sequence method, we showed that human DC can be loaded successfully with fusion peptides incorporating MART-1_{27-35} (122). We found that the addition of signal sequence at the N-terminus, but not at the C-terminus, of this epitope greatly prolonged its presentation in DC. A newly designed peptide construct, composed of the MART-1_{27-35} epitope replacing the hydrophobic part of a natural signal sequence, was also effective. Interestingly, as with our earlier work with T2 cells, an artificial signal sequence containing the epitope was the most efficient construct for enhancing its presentation. These findings may be of practical significance for the development of synthetic anticancer vaccines and *in vitro* immunization of CTL for adoptive immunotherapy. Although viral vectors are efficient vehicles for gene transfer into DC, non-viral delivery of antigens has its advantages too. Fusion peptides can be readily produced in large quantities, and are very stable. In addition their application is not associated with immune responsiveness to vector-derived immunogens, or with risk of recombination.

The encouraging results in experiments in mice, as well as improved techniques for *in vitro* immunization and expansion of DC, support the initial attempts to immunize patients with DC expressing tumor antigens.

Development of an efficient method for isolation and partial purification of DC (123) led to the infusion of antigen-pulsed DC into four patients with follicular low-grade B cell lymphoma (124). Complete remission was observed in two patients, one patient had a partial response, and one patient had stable disease. In contrast, immunization with the antigen (monoclonal surface immunoglobulin) alone or emulsified in adjuvants did not induce regression of lymphoma (125).

Another clinical study showed that DC pulsed with idiotypic protein derived from serum in patients with multiple myeloma induced a specific CTL response in one patient (126). Bohlen *et al.* vaccinated patients with relapsed myeloma using DC pulsed with idiotypic protein-derived peptides (127). Specific T-cell responses were not reported, although 3 of 7 patients developed humoral anti-idiotypic responses following vaccination. Wen *et al.* (128) reported the vaccination of a single myeloma patient with idiotype-pulsed monocyte-derived DC. Anti-idiotypic T-cell proliferative and cytotoxic responses were measurable following vaccination but no significant clinical response was observed. More recently, Lim *et al.* immunized 6 patients with multiple myeloma with idiotypic protein-pulsed DC (129). PBMC proliferative responses to the idiotypic protein were observed in 5 of the 6 patients following treatment. There were also increases in CTL precursor frequencies for idiotypic protein-pulsed autologous targets in 3 patients. Idiotypic protein-pulsed DC vaccination can therefore elicit potentially useful anti-myeloma immune responses in patients with multiple myeloma.

Antimelanoma CTL were generated *in vitro* from healthy donors (130) and melanoma patients (131) with DC pulsed with melanoma-derived peptides. It was also shown that vaccination of patients with melanoma with DC pulsed with MAGE-1-derived peptide elicited melanoma-specific CTL *in vivo* (53). In another clinical study, 16 melanoma patients were immunized with peptide-pulsed or tumor lysate-pulsed DC (132). Vaccination was well tolerated in all patients. Objective clinical responses were observed in 5 out of 16 patients with regression of metastases in various organs. In a similar trial, six HLA-A2+ patients with metastatic melanoma received 4 weekly injections with monocyte-derived DC pulsed with HLA-A2-restricted peptides derived from MART-1, gp100, and tyrosinase (133). Complete regression of a subcutaneous mass lasting more than one year has been observed in one patient. Mature, monocyte-derived DC pulsed with MAGE-3 A1 peptide were used to immunize 11 far advanced stage IV melanoma patients (134). Significant expansions of MAGE-3 A1-specific CTL precursors were induced in 8 of the 11 patients. Regressions of individual metastases were observed in 6 of the 11 patients. In a recent phase I study, 14 melanoma patients received i.v. infusions of DC pulsed with a pool of either HLA-A1-restricted peptides MAGE-1 and MAGE-3 or HLA-A2-restricted peptides MART-1, gp100, and tyrosinase (135). Clinical and immunological responses consisted of tumor regressions in 2 patients, increased melanoma peptide-specific DTH reactions in 4 patients, significant expansions of MART-1 and gp100-specific CTL in one patient, and development of vitiligo in another HLA-A2+ patient. These studies prove that DC vaccines can elicit tumor specific CTL and cause regressions even in advanced malignant melanoma.

Several prostate tissue-associated antigens are now being explored as targets for prostate cancer immunotherapy: prostatic alkaline phosphatase (PAP), prostate-specific membrane antigen (PSMA), and PSA. Valone *et al.* (136) have carried out a trial of partially purified peripheral blood DC pulsed with recombinant PAP protein in 12 patients with advanced prostate cancer. Intravenous administration of peptide-pulsed DC monthly for 3 months resulted in T-cell proliferative responses to PAP in all patients. In a phase II trial, involving infusions of autologous DC and two human HLA-A2-specific PSMA peptides, 30% of the participants were identified as clinical responders (137). In the follow-up evaluation of 19 responders in two study groups (metastatic group and local recurrence group), the average duration of response was 149 days for the metastatic group, and 187 days for the local recurrence group. The same researchers recently reported the immune monitoring of a phase II clinical trial in prostate cancer patients before and after immunotherapy with DC exogenously pulsed with two PSMA-derived peptides (138). Clinical responses were strongly associated with 2 indicators of immunocompetence: skin test responses to recall antigens and cytokine secretion by T cells after non-specific stimulation. These authors also reported, that infusions with PSMA-pulsed DC can be given with greater numbers of DC and a lesser number of infusions, with no loss of response rates. (139).

Schott *et al.* reported induction of cellular immunity in a patient with parathyroid carcinoma treated with tumor lysate-pulsed DC (140). The patient was immunized with tumor lysate and parathyroid hormone-pulsed DC. Antigen-loaded DC were delivered by subcutaneous and intralymphatic injections. After 10 vaccinations, a specific cellular immune response to tumor lysate was observed. Reiser *et al.* performed a pilot study with a DC-based vaccine in 4 patients with advanced renal cell carcinoma (141). DC were loaded with autologous tumor cell lysate plus KLH and matured with a combination of TNF-α and prostaglandin-E2. This approach resulted in the induction of T-helper type-1-dominant immune responses in these patients.

These clinical trials suggest that several types of cancer may be responsive to DC-based immunotherapy. However, the source of the DC for vaccination and the frequency of the CTL precursors in cancer patients should be carefully evaluated. In patients with a low frequency of peptide-specific precursors, the efficient activation of antigen-specific CTL required the use of peptide-loaded CD34+-derived, but not monocyte-derived, DC (142). This suggested that DC derived from CD34+ cells and monocytes were not functionally equivalent for the activation of CTL in patients with a low CTL precursor frequency.

Heat shock proteins

An interesting approach in vaccine development is the use of heat shock protein-peptide complexes for vaccination. Heat shock proteins (HSP) derived from any given cell type associate with a wide variety of peptides generated during

protein degradation (143). Vaccination of mice and rats with HSP-peptide complexes has resulted in powerful immune responses against the peptides bound to HSP, but not to HSP itself (144). More recently, Yedavelli *et al.* reported that tumor derived HSP-peptide complexes can be used as an effective prophylactic and therapeutic agent even in poorly immunogenic cancer such as prostate cancer (145). So far, 4 classes of HSP preparations: gp96, HSP90, HSP70, and calreticulin have been used successfully to immunize against cancer and infectious diseases in prophylactic and therapeutic protocols (146). However, since tumors are not always available in large quantity, it is difficult to isolate HSP from the same tumor sample. Menoret *et al.* developed a new method for preparation of multiple HSP-based vaccines from a single tumor sample in one step using heparin-agarose chromatography (147). Two recent studies reported significant enhancement of DNA vaccine potency by linkage of antigen genes to HSP genes (95) (148). Both studies utilized human papillomavirus type 16 E7 as a source of model antigen and reported increased potency of the new vaccine against E7-expressing tumors. A disadvantage of the HSP approach in clinical settings is the requirement for generation of customized, patient-specific vaccines for cancer. However, an attractive feature of the vaccinations with HSP-peptide complexes is their ability to elicit specific CTL response in mice of any haplotype. This may be because the association of peptides with HSP occurs before their association with the MHC molecules, and therefore, these peptides are not associated with any particular haplotype.

Adjuvants

Vaccines derived from tumor cells or tumor lysates were used before the identification of tumor associated antigens (149) (150). Various adjuvants were important to improve the efficiency of these vaccines in eliciting polyvalent antitumor immune responses. Since a main disadvantage of peptide-based vaccines is their low immunogenicity, careful selection of the adjuvants used with these vaccines is even more important. The function of each adjuvant depends on its ability to affect the pathway of the antigen presentation, to prolong the antigen exposure to the APC, as well as to influence the number and the type of the APC and the release of cytokines in the local environment. New adjuvants currently being developed include preparations that resemble incomplete Freund's adjuvant, preparations derived from bacterial cell wall and modified in order to reduce toxicity, and different cytokines (80). Use of cytokines as vaccine adjuvants is very attractive since it may allow precise modulation of the direction of the immune response. Since granulocyte/macrophage colony-stimulating factor (GM-CSF) plays an important role in the maturation and function of APC such as DC and macrophages, it has attracted attention as a cytokine adjuvant (151). Combination therapy with GM-CSF and IL-4 in patients with metastatic solid malignancies was reported to enhance the number and antigen-presenting ability of circulating of CD14+ and CD83+ cells (152). Another study found that treatment of cancer patients with GM-CSF and TNF-a increased the number of DC in the skin and the number of DC-precursors in the blood of some patients with cancer (153). Leong *et al.* found that GM-CSF injected with autologous melanoma vaccine mediated tumor regression in patients with metastatic melanoma (154). The use of other cytokines like IL-2, IL-10, and IL-12 as vaccine adjuvants was reported. The efficacy of recombinant poxvirus-based vaccines was greatly improved by the addition of exogenous IL-2 to the vaccination regimen (155). Rosenberg *et al.* noticed that immunization of melanoma patients with gp100-derived peptide plus IL-2 improved the clinical response to the vaccine (156). At the same time, a decrease in immune reactivity was observed when peptide was administered with IL-2. The authors thus suggested that immunization with peptide plus IL-2 resulted in sequestering or apoptotic destruction of newly activated immune cells at the tumor site. IL-12 is also a promising vaccine adjuvant, because of its role in directing Th1 responses and, thus, CTL responses (157). In a phase I trial with recombinant human IL-12, injected subcutaneously or intralesionally in patients with cutaneous T cell lymphoma, it was found that this cytokine augmented the antitumor CTL responses and caused tumor regressions in some patients (158). These studies suggest that the cytokines and other adjuvants are an important component of the optimal vaccine formulations.

Future directions

The growing number of human tumor antigens identified becomes a solid basis for cancer vaccine development. However, the antigenic profile of human tumors is very complex, and consists of many peptides originating from various classes of proteins. This fact should be considered carefully in designing cancer vaccines. An important question is which tumor antigens are the most important in tumor regression *in vivo*. Differentiation antigens may play an important role in tumor regression, which is suggested by the positive correlation between the development of vitiligo and a good clinical response to immunotherapy in melanoma patients. Promising candidates are also MAGE, BAGE, and GAGE antigens since they are expressed in a variety of cancer cells, but not in normal cells except testis. Mutated epitopes such as CDK-4 and b-Catenin are tumor specific, but immunotherapy using these antigens is likely to be applicable only to individual patients. The characterization of class II-restricted antigens as targets for CD4+ T cell responses will allow concurrent immunization with class I and class II epitopes in order to generate more potent immune responses. In any case, the ideal vaccine will most likely consist of a cocktail of tumor antigens or proteins. One must also consider the dose of antigen and the speed of antigen release in the vaccine formulations. High doses of antigen released

faster may induce T-cell tolerance (159). Immune tolerance may be due to fast expansion and subsequent elimination of specific T-cell clones, or to apoptosis induced by repeated stimulation of already stimulated T-cells in cell cycle (160). Therefore, it is essential to select as immunogens those epitopes against which tolerance has not been induced (161).

Future cancer vaccine strategies will most likely focus on more potent approaches for immunization. The use of the entire antigenic proteins might well be superior to peptide vaccines. A whole protein may provide several T-cell epitopes presented by different MHC molecules, which is the case with tyrosinase (presented by HLA-A2 and HLA-A24), MAGE-1 (presented by HLA-A1 and HLA-Cw16) and MAGE-3 (presented by HLA-A1 and HLA-A2). An additional advantage of the whole protein vaccines may be the induction of humoral immune responses (162).

Many challenges exist in the development of safe and effective vaccines for cancer. Cancer cells can undergo genetic alterations that result in loss of antigen expression or loss of the ability to present the tumor antigens. Recent advances in the design of polyvalent vaccines targeting several antigens may solve this problem. In addition, the possibility to treat patients with vaccines earlier in the course of the disease and to combine vaccines with other treatment modalities may also improve the vaccine efficacy. Ultimately, multiple vaccine strategies, applied in synergy, will most likely be responsible for the future success of cancer immunotherapy.

Acknowledgements

This work was supported by a grant from the Pfeiffer Foundation

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Received June 13, 2000
Accepted July 10, 2000

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nc - QR180.I52

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IMMUNOLOGICAL INVESTIGATIONS, 29(2), 131–137 (2000)

Heat Shock Proteins and Cancer Immunotherapy

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ABSTRACT

Vaccination with heat shock proteins from tumor have been shown to elicit an anti-tumor response. Current studies indicate that the immunogenecity of HSPs is derived from the antigenic peptides which they associate with. Mechanisms by which the HSP-peptide complexes induce an immune response and the possible role of HSPs in antigen presentation is discussed in this article. The use of HSP-peptide complexes can be used as tumor vaccines for cancer immunotherapy is reviewed.

Heat shock proteins (HSPs) were first recognized as a set of polypeptides induced in *Drosphila* by elevated temperatures. They are highly conserved and abundant proteins in both eukaryotes and prokaryotes (1). Heat shock proteins are divided into several major families, Hsp110, 90, 70, 60/GroEL and the small HSPs based on their size and structure (2, 3, 4). HSPs have been found to be induced by environmental stress (e.g. heat shock, ethanol, heavy metal, glucose deprivation, inhibitor of glycosylation), pathological stress (viral infection, inflammation, fever and tissue trauma) (5) and even non-stressful conditions (cell cycle, cell differentiation and development) (6). Many HSPs also function as molecular chaperones that prevent irreversible aggregation and assist protein folding, unfolding, assembly and transport. These functions are based on the abilities of HSPs to bind unfolded peptide chains. The heat shock proteins are primarily localized in the cytoplasm and nucleus. A parallel set of stress proteins which are differentially inducible (e.g. by anoxia) are called GRPs and are localized in the endoplasmic reticulum. Primary GRPs fall into families parallel to the major HSPs: i.e. grp78, grp94(gp96) and grp170. Recently, HSPs and GRPs derived from tumors were shown to be able to protect mice against the subsequent challenge with tumor from which the HSPs were

purified. This has been shown to be related to the general peptide binding properties of HSPs. Because of these findings, the role of str ss proteins in tumor immunology has attracted significant attention.

Tumor-derived HSPs elicit protective immunity against cancers

In the early 1990's, Srivastava and his colleagues first found that a tumor rejection antigen, isolated by biochemical fractionation of tumor cells, was a heat shock protein (grp94/gp96) (7, 8, 9, 10). There is unequivocal evidence today that the HSPs, including hsp70, hsp90 and grp94/gp96, derived from a given cancer, can elicit protective immunity specific to that particular cancer. HSPs derived from normal tissues do not protect against any cancer tested. In the last several years, immunogenecity of HSP preparations from tumors has been repeatedly seen in different experimental tumor systems of distinct histological origins, which range from chemical or UV-radiation induced tumors to spontaneous tumors (11, 12, 13). Furthermore, two high-molecular-weight heat shock proteins, hsp110, grp170 derived from CT26 and MethA tumors (Wang et al., unpublished data) were recently found to induce an anti-tumor response against these tumors. Most importantly, it has been shown that the immunization of mice with gp96 resulted in the induction of memory T cells (14). Based on these observations, it is apparent that HSPs prepared from tumor are able to act as tumor vaccines.

Immunogenicity of HSPs is due to their peptide-binding properties as chaperones

Molecular cloning and sequence studies indicated that the genes coding for HSPs in the tumor cells and normal tissues did not exhibit any difference in nucleotide sequence (9). It was hypothesized that immunogenicity of HSPs lies not in the HSPs isolated from tumor or normal tissues, but rather in the peptides that they bind (8).

Studies of HSP structure and chaperoning properties by molecular biologists have provided significant evidence for peptide binding activities of heat shock proteins. It was shown that peptide-binding sites consisting of several β sheets exist in heat shock protein 70 (15). Furthermore, hsp70 association with peptides has been demonstrated in vitro (16, 17). Although the peptide-binding structure for gp96 is unknown, peptide-binding activities of gp96 have also been demonstrated. gp96 has been shown to transfer peptides from the transporter associated with antigen processing (TAP) to MHC class I molecules (18, 19, 20). Recently, TAP-independent peptides were also found to bind gp96 (21). In our laboratory, analysis of secondary structure indicated that, while exhibiting similarities to hsp70, hsp110 and grp170 appear to exhibit peptide-binding clefts with a significantly enlarged "lid" domain. This suggests that hsp110/grp170 binding affinities and/or capacities differ from hsp70. Recent studies have

confirmed that hsp110 exhibits a different peptide-binding capacity (22, 23). While little is known about GRP170 functions, it is evident that it is involv d in binding immunoglobulin chain in the endoplasmic reticulum (ER) (24). Most notably, GRP170 may be the ATPase responsible for peptide import into the ER from TAP (18, 25, 26).

Recent studies from different laboratories also provide convincing evidence for the binding of antigenic peptide as the basis of the immunogenic activities of HSPs using a number of well-defined systems. Immunization with gp96 isolated from vesicular stomatitis virus (VSV) infected cells primed VSV-specific cytotoxic T lymphocytes (CTL) (27). Similarly, the gp96 isolated from β-galactosidase (β-gal) transfected cells elicited CTLs specific for β-gal and minor histocompatibility antigens expressed in these cells (28). This hypothesis is also directly supported by the observation that peptide-depleted hsp70 by ATP treatment was unable to elicit immunity against tumor challenge (29, 30). Most importantly, hsp70-peptide and gp96-peptide complexes can be reconstituted in vitro, and these complexes can induce peptide-specific CTLs (31). Young and colleagues also reported that immunization with recombinant hsp70-OVA fusion proteins protected mice against challenge with an OVA-expressing tumor (32). In contrast, fusion proteins not containing hsp70 were ineffective. All of these studies and others indicate that HSP-chaperoned peptides are responsible for the antigen specific immune response. Thus, HSPs have been suggested to be the first physiological mammalian adjuvant and they may be used as an antigen delivery vehicle for immunotherapy (31).

Mechanism of HSP-peptides elicited immune response

The mechanism through which immunization with HSP-peptide complex elicits antigen-specific CD8+ T cells is being worked out by a number of investigators. By using macrophage and T cell depletion studies, Udono et al demonstrated that this priming of the immune response by Hsp-peptide complex was sensitive to the functional abrogation of phagocytic cells (33). Macrophages were shown to internalize gp96-peptide complex and re-present the gp96-chaperoned peptide on the MHC I molecules (34). It was also found that peptide was actually recycled through a nonacidic compartment in the cell and not simply transferred to MHC class I molecule on the cell surface directly. Most interestingly, it seems that HSP-chaperoned peptides are independent of the MHC-type of the tumor from which they are derived, whereas, their presentation to the CTL is defined by the MHC phenotype of the APCs (34). Recently, it has been shown that immunization with bone morrow generated dendritic cells which were pulsed with tumor-derived HSPs elicited an anti-tumor response, suggesting that antigen presenting cells (APC) are critical for HSP-peptide complex mediated immune responses (Wang et al unpublished data). Furthermore, hsp70 released from tumor cells was seen to be internalized

directly into DCs and enhanced the capability of DCs to take up proteins/peptides, indicating that in addition to the function of chaperoning antigenic peptides from tumor, heat shock proteins themselves might act as a messenger to deliver an immunological signal to the host system (35). However the details of the intercellular events involved in the transfer of HSP-chaperoned peptides onto MHC class I molecules remains unknown.

Suto and Srivastava demonstrated that brefeldin A inhibited the presentation of HSP-chaperoned peptides (34). This study suggested that transport between endoplasmic reticulum (ER) and Golgi apparatus is necessary for the antigen re-presentation pathway. KDEL receptors recycle between Golgi and ER, thereby retrieving resident ER proteins that escaped from the ER (36). Also exogenous toxins such as Psedomonoas exotoxin and ricin are transported from Golgi to ER by interacting with KDEL receptors (37, 38). Thus, whether or not exogenous HSPs, especially ER resident GRPs, require KDEL receptors for their retrograde transport is an interesting possibility. In addition, the high efficiency of small quantities of HSPs to elicit an immune response indicates that there may exist HSP receptors on the surface of antigen presenting cells which are capable of taking up HSP-peptide complexes specifically. Arnold-Schild et al provided supporting evidence showing that gp96 and hsp70 bind specifically to the surface of APCs and are internalized spontaneously by receptor-mediated endocytosis. Furthermore, internalized HSPs were observed to co-localize with surface MHC class I molecules in early and late endosomal structures, indicating that HSPs are involved in the processes of antigen presentation (39) The HSP receptor may take up the HSP-peptide complex in a manner similar to that used for antigen uptake by the mannose receptor or the FCγ receptor, which are also expressed on dendritic cells (40, 41, 42). Whether the uptake of HSPs is a specific or non-specific process still requires further study. Identification of the receptors responsible for the internalization of HSPs would contribute significantly to our understanding of the mechanism of the HSP-peptide complex elicited immune response.

Potential of using HSP as tumor vaccines

Each cancer has a specific antigenic fingerprint which consists of a large repertoire of mutated or non-mutated peptides (43). HSP vaccines are unique because of their ability to chaperone and represent a broad antigenic repertoire of tumor cells. Thus, vaccination with HSPs isolated from tumor cells circumvents the need to identify specific tumor antigens, and hence extends the use of HSP-based immunotherapy to the majority of cancers where specific tumor antigens have not yet been characterized. Moreover, since HSP vaccines are directed against the entire antigenic repertoire of that tumor, this avoids the possibility of immunological escape. All these studies suggest a promising futur for HSPs as cancer vaccines.

Many HSPs are believed to be located in intracellular compartments, but cytosolic HSPs were recently found to be present on the cell surface and to be involved in the anti-tumor response. These HSPs seem to function as a target structure which can be recognized by γδ T cells and NK cells (44, 45, 46, 47). More interestingly, it was reported that the recognition of hsp70 on the target cells can be blocked by anti-hsp70 antibodies, but not by anti-MHC class I antibodies or anti-NK antibodies (48, 49). The endoplasmic reticular HSP grp94/gp96 was also seen to localize on the cell surface of tumor cells (50) and exposure of gp96 to microphages resulted in the secretion of a low level of cytokines, regardless of the peptides which gp96 binds (34). These observations are consistent with the idea that HSPs might act as antigen-presenting molecules themselves and possibly HSPs are involved in both antigen-specific and antigen-nonspecific immune responses. However, the mechanisms of HSP surface expression and its roles in the immune response still require further investigation. It is also conceivable that HSP-based immunotherapy may not only promote T cell-dependent anti-tumor immunity but also directly induce NK cell activation in vitro. Indeed, HSPs may be involved in the interaction between adaptive and innate immune responses.

It has been known that heat shock proteins not only protect cells from heat, but also render cells resistant to cell death induced by oxidative stress, TNF, and chemotherapeutic drugs (51, 52, 53). All of these data suggest that HSP expression in the tumor could enhance tumorigenesis and limit the efficacy of cancer therapy (54, 55). In addition, although HSP expression was recognized as a prognostic value in certain tumors, the data are limited and the results are contradictory (56, 57). Consistent with the observations that immunization with tumor-derived HSPs elicited tumor-specific immunity, it has been shown that immunogenicity of tumor cells co-segregate with the expression of heat shock proteins (58). Stable transfection of autologous HSP70 in tumor cells significantly enhances the immunogenicity of tumor, suggesting that increased levels of HSP may provide an immunostimulatory signal in vivo which helps break tolerance to tumor antigens (59). Based on the observations described above, heat shock proteins seem to play multiple functions in the tumor cell. It could increase the immunogenicity of tumor cells, while it could also help the tumor cell survive. Several questions arise: Is the high expression of HSP in the tumor a good or bad prognostic indication? What is the role of heat shock proteins in the tumor immunogenicity? Is it possible to manipulate tumor immunogenicity therapeutically if HSP expression correlates to immunogenicity of tumor? Do all HSP members perform similar immunological functions in the tumor? Can they all be used as tumor vaccines?

Collectively, the capability of HSPs to chaperone antigenic peptides and induce CTL has profound immunological implications. Although many questions remain unanswered, there is now unequivocal vidence from many laboratories that heat shock proteins (HSPs) can serve as

vaccines. Further studies of the physiological and immunological roles of HSPs in cells, including tumor cells, will help the translation of HSP-based immunotherpay into a new generation of anti-cancer vaccines against cancers.

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Thank you.

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Biochemical Pharmacology, Vol. 58, pp. 1381–1387, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00178-1

COMMENTARY

Stress Protein/Peptide Complexes Derived from Autologous Tumor Tissue as Tumor Vaccines

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ABSTRACT. Vaccination of inbred mice with tumor-derived stress proteins hsp70, hsp90, and gp96/grp94 elicits a protective immunity to the tumor from which the vaccine was purified. There is now comprehensive experimental evidence that the antigenicity of tumor-derived hsp70, hsp90, and gp96 preparations results from diverse arrays of endogenous peptide antigens complexed with these stress proteins. Vaccination with tumor-derived stress protein/peptide complexes leads to their uptake and processing by professional antigen-presenting cells and to presentation of associated tumor peptide antigens to cytotoxic T cells. This induces a tumor-specific cytotoxic T cell response. The attractiveness of the concept of using tumor-derived stress proteins as vaccines is derived from two observations: (i) tumor stress protein vaccines mirror the individual antigenicity of a tumor, which results from random mutations due to genetic instability; and (ii) stress proteins represent powerful adjuvants for the peptide antigens complexed to them. BIOCHEM PHARMACOL 58;9:1381–1387, 1999.

KEY WORDS. tumor immunology; heat shock proteins; T lymphocytes; tumor antigens; tumor vaccines; gp96

The induction of immunological control over malignant tumors by vaccination is the major research aim in tumor immunology. An important part of this research focuses on the activation of a specific T cell response against tumors. It is possible to induce a transplantation immunity in inbred mice that is strictly specific for the tumor that was used for vaccination [1–5]. By adoptive transfer experiments and *in vivo* depletion experiments it was shown that this tumor-specific transplantation immunity is mediated by T cells [6]. The growing knowledge of the molecular biology of cancer cells shows that cancer cells express a multitude of altered proteins due to mutations in the course of carcinogenesis and to the genetic instability of cancer cells. These alterations should be recognized by T cells of the tumor-bearing host as altered peptide epitopes, because it can be assumed that the host has not developed thymic tolerance toward these alterations. However, tumor development and growth are not under immunological control because tumor cells cannot efficiently activate T cells. In contrast to professional APC†, tumor cells do not express costimulatory molecules or a supportive cytokine milieu, which is necessary for efficient T cell activation [7]. On the contrary, tumor cells often secrete immunosuppressive cytokines such as transforming growth factor β, which promotes immune escape of the tumor [8], express apoptosis-inducing signals for T cells such as the CD95 ligand [9], and interfere with signal transduction in T cells [10]. Modern vaccination strategies against tumors try to circumvent these immunosuppressive mechanisms of tumor cells. One of these strategies is vaccination with subcellular material from tumor cells, which mirrors the individual antigenicity of the respective tumor. This approach employs professional APC for the induction of immunity. An autologous tumor vaccine should be manufactured from tumor cells by a standardized method and ideally should consist of defined cellular proteins. Experimental evidence shows that cellular stress proteins such as hsp70, hsp90, and gp96/grp94 and the endoplasmic chaperone calreticulin are complexed in cells with a diverse array of peptides including immunogenic peptide antigens [11–14] (Fig. 1). If these stress protein/peptide complexes are derived from tumor cells, they should represent at least a part of the individually distinct antigenic repertoire of the tumor. In consequence, they could be used as autologous tumor vaccines. In the following commentary, the experimental evidence for this concept of tumor-derived stress proteins as autologous tumor vaccines will be presented.

STRESS PROTEINS AS CHAPERONES FOR ENDOGENOUS PEPTIDES

Stress proteins are among the most abundant proteins in cells under physiological conditions because of their essential role as chaperones involved in protein transport and folding. Most stress proteins are expressed constitutively. Their expression can be enhanced under stress conditions

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† *Abbreviations:* APC, antigen presenting cells; CTL, cytotoxic T lymphocytes; hsp, heat shock protein; LCMV, lymphocytic choriomeningitis virus; MHC I, major histocompatibility complex class I; SV40, simian virus 40; TAP, transporter associated with antigen presentation; and VSV, vesiculostomatitis virus.

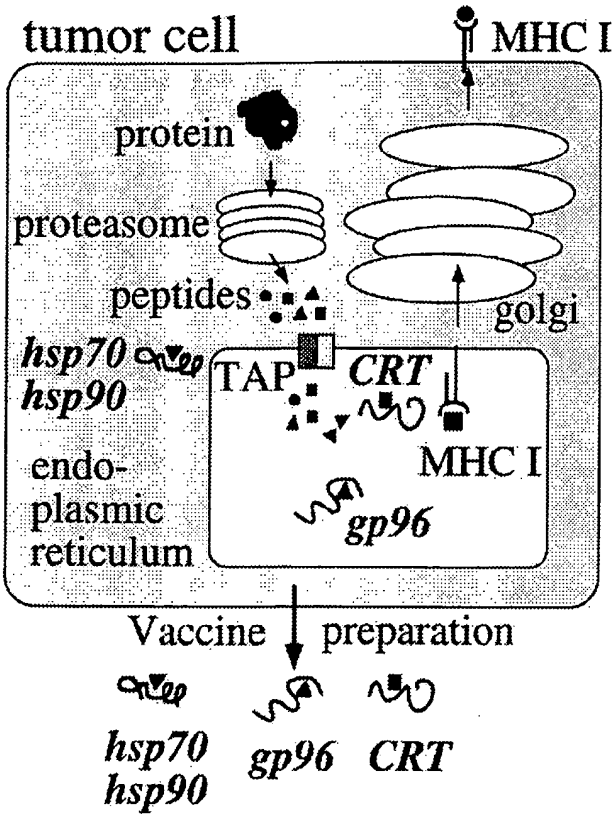

FIG. 1. Concept of tumor-derived stress proteins and chaperones hsp70, hsp90, gp96, and calreticulin (CRT) as autologous tumor vaccines. There is now comprehensive experimental evidence that the antigenicity of tumor-derived hsp70, hsp90, gp96, and calreticulin is derived from diverse arrays of endogenous peptide antigens complexed with these stress proteins. These peptide antigens are produced by proteasomal degradation of cytoplasmic proteins and can be complexed in the cytoplasm to hsp70 or hsp90. When peptide antigens are transported by TAP molecules into the endoplasmic reticulum, they either can directly associate with MHC class I molecules or can be bound by endoplasmic stress proteins or chaperones such as gp96 and calreticulin.

such as heat shock or glucose deprivation. Other stress proteins are inducible by stress conditions. There are several lines of evidence for the peptide binding functions of stress proteins hsp70, hsp90, and gp96, which are described below.

(i) The peptide binding function of stress proteins can be explained well by their functions under physiological and stress conditions. Under physiological conditions, stress proteins prevent immature folding of nascent proteins, assist intracellular protein transport through membranes, guide the assembly of protein complexes, and regulate function and degradation of cellular proteins. Under stress conditions, stress proteins prevent aggregation of partially denatured and misfolded proteins [15–18]. Due to these functions, stress proteins must have the ability to bind promiscuously to exposed peptide sequences of partially unfolded, misfolded, or nascent proteins.

(ii) The structures of stress proteins reveal possible peptide binding sites. Recently, the existence of a peptide binding pocket has been demonstrated in the hsp70 crystal structure [19]. Evidence for two substrate binding sites has been obtained for hsp90 [20].

(iii) Isolated stress proteins were shown to bind peptides *in vitro*. Hsp70 binds and releases peptides by cycles of ATP hydrolysis and ATP binding [21, 22]. Gp96 binds peptides independently of ATP hydrolysis in a temperature-dependent fashion, most likely by conformational change [23, 24]. Hsp70 and gp96 could be complexed with immunogenic peptide antigens *in vitro*. The reconstituted stress protein/peptide complexes elicited a peptide-specific CTL response in mice [23, 25].

(iv) Stress proteins were shown to bind peptides under physiological conditions. It was demonstrated recently by peptide translocation assays that gp96 is one of several peptide binding chaperones of the endoplasmic reticulum (ER) besides protein disulfide isomerase and calreticulin [26–28]. The peptide translocation in these studies was dependent on the function of TAP. Another study showed, by vaccination experiments with gp96 prepared from TAP-deficient or TAP-competent cells, that gp96 may associate with peptides whose presence in the ER is either TAP-dependent or TAP-independent [29]. Three studies obtained direct evidence for the association of naturally processed CTL-recognized peptide epitopes with hsp70 and gp96 in cells. Nieland *et al.* [30] reported the isolation of a naturally processed VSV-peptide epitope from gp96 preparations of VSV-infected cells, and Breloer *et al.* [31] showed the isolation of naturally processed ovalbumin epitopes from gp96 and hsp70 derived from ovalbumin-transfected cells. Ishii *et al.* [32] reported the isolation of an individual tumor peptide antigen from preparations of stress proteins hsp70, hsp90, and gp96 derived from a murine fibrosarcoma.

TUMOR STRESS PROTEINS AS TUMOR-SPECIFIC TRANSPLANTATION ANTIGENS

The important basis for the concept of tumor stress proteins as autologous tumor vaccines was the identification of stress proteins hsp70, hsp90, and gp96 as tumor-specific transplantation antigens of chemically induced mouse tumors [12, 33–35]. It was observed that the vaccination of inbred mice with tumor stress proteins elicited a strictly tumor-specific transplantation immunity against the syngenic tumor from which the stress proteins were derived. This individually distinct tumor immunity induced by tumor stress protein vaccination can be explained by an individually distinct array of immunogenic peptide antigens complexed with stress proteins in each tumor, since polymorphisms of stress proteins hsp70, hsp90, and gp96 are not known [36].

It was demonstrated that the priming phase of the immunization with tumor-derived gp96 was dependent on $CD8^+$ T cells and macrophages [37]. Immunization of mice with gp96 derived from UV-induced syngenic tumors elic-

ited tumor-specific CTL in parallel with tumor-specific transplantation immunity [38]. This suggests a model in which the vaccination with tumor-derived stress protein/peptide complexes leads to cross-priming of tumor-specific CTL by phagocytic APC. These APC must be able to take up and process the stress protein/peptide complexes and channel the peptide antigens into the MHC class I restricted presentation pathway.

The tumor immunity that is induced by tumor stress protein vaccination was demonstrated initially by preventive immunization against tumors. This seems not to be applicable in humans in view of the individuality and multiplicity of tumor antigens. However, it was shown recently that vaccination with tumor-derived gp96 and hsp70 also had therapeutic effects in early tumor growth and metastasis [39, 40]. So far, the applicability of tumor stress proteins as tumor vaccines has not shown restrictions concerning the type of experimental tumor, the histological origin, or the species. Tumor immunity induced by tumor-derived stress proteins was demonstrated against chemically induced tumors, UV-induced tumors, and spontaneous tumors of different histologic origins such as fibrosarcomas, lung carcinomas, melanomas, colonic cancers, and prostate cancers in mice and rats [33, 34, 38–41].

ACTIVATION OF ANTIGEN-SPECIFIC CTL BY ENDOGENOUS GP96/PEPTIDE COMPLEXES

In different antigenic systems the immunization of inbred mice with gp96 preparations elicited CTL responses against antigens of the cells from which gp96 was derived. This was shown for viral antigens from SV40 [42] and VSV [43], for influenza virus nucleoprotein [44], and for CTL-recognized model antigens such as β-galactosidase and minor histocompatibility antigens [45]. We investigated whether gp96 derived from human melanoma cells is associated with different CTL-recognized melanoma peptide antigens. It could be demonstrated that autologous human CTL clones specific for different melanoma peptide antigens were stimulated preferentially by gp96 derived from autologous melanoma cells compared with gp96 derived from autologous B cells. The CTL stimulation was dependent on monocytes or dendritic cells as APC. These results confirm for the first time in a human tumor model the association of gp96 with CTL-recognized tumor peptide antigens.*

There is no evidence that the spectrum of peptides associated with gp96 is dependent on the MHC haplotype of the cell. Therefore, gp96 as well as stress proteins hsp70 and hsp90 should be associated with peptides or precursors of peptides that differ in their binding motifs for MHC molecules and possibly can be presented by allogenic MHC molecules. This was confirmed by the observation that $CD8^+$ T cells of H-2b mice could be primed against minor H antigens by gp96 purified from H-2d positive cells [45], and VSV-specific CTL were induced in H-2b mice by gp96 preparations from H-2d VSV-infected cells [43]. This cross-priming potential by gp96 is important in view of shared tumor antigens, such as many CTL-recognized melanoma antigens. Individuals with different MHC haplotypes could possibly be cross-primed against shared tumor antigens by gp96 preparations from allogenic cell lines expressing these shared antigens.

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ADJUVANT FUNCTION OF STRESS PROTEINS

Recently, it was shown that vaccination with *in vitro* reconstituted complexes of peptide antigens with gp96 or with hsp70 induces peptide-specific CTL responses and protective immunity *in vivo*. The induction of this immunity is dependent on binding of the peptide to the stress protein and was not induced by vaccination with peptide alone or mixtures of stress protein and peptide. Blachere *et al.* showed for different peptide antigens (viral and nonviral CTL epitopes) that vaccination with gp96/peptide complexes and hsp70/peptide complexes induced a peptide-specific CTL response, whereas vaccination with peptides alone or non-complexed mixtures of stress proteins and peptides did not. Additionally, the immunization with gp96/VSV-peptide complexes induced a protective immunity against a challenge with VSV-transfected tumor cells [23]. Ciupitu *et al.* showed that vaccination with hsp70/LCMV peptide complexes elicited LCMV-specific CTL and protective immunity against LCMV [25]. Vaccination with fusion proteins consisting of stress proteins and protein antigens seems to be an additional way to elicit antigen-specific CTL responses [46]. The mechanism of this adjuvant effect mediated by stress proteins is still unclear. Specific receptor-mediated uptake of stress protein/antigen complexes and primary activation of antigen-presenting cells by hsp70 and gp96 may play a role [14, 36, 43]. It is known from earlier work that hsp70 and hsp65 can serve as powerful adjuvants for protein and carbohydrate antigens in eliciting helper and humoral responses [47–51]. However, the recent work shows that stress proteins are natural adjuvants which channel bound antigens into the MHC class I presentation pathway, a very important observation for the development of vaccines against diseases that can be cleared by a cytotoxic T cell response. The attractiveness of stress proteins as adjuvants in contrast to conventional adjuvants lies in their lack of toxicity and in their potency to amplify both helper and cytotoxic T cell responses.

STRESS PROTEIN EXPRESSION IN TUMORS

Stress proteins confer on tumor cells an increased resistance against stress conditions such as hypoxia, acidosis, and glucose deprivation [52]. In consequence, overexpression of stress proteins by tumors is more likely than weak or lost expression. This hypothesis is confirmed by studies showing overexpression of constitutively expressed or inducible

stress proteins of the hsp70 and hsp90 family in tumor tissue [53–55]. We found clear overexpression of gp96 in colorectal cancer cells compared with tumor stroma in 34 of 51 colorectal primary tumors and similar expression in cancer cells and stroma in the remaining 14 colorectal cancer specimens tested by immunohistochemistry. No loss of gp96 expression was observed in liver (N = 20) and lymph node metastases (N = 20). The transcription of gp96 in colorectal cancer cell lines was enhanced by glucose deprivation, not by heat shock, confirming the role of gp96 as a glucose-regulated stress protein in human colorectal cancer (Heike *et al.*, unpublished data). These results point to an essential role of gp96 in colorectal cancer cells in protection against hostile conditions of the tumor microenvironment, such as glucose deprivation. The protective role of tumor stress proteins is in agreement with the observation that increased expression of hsp70 and gp96 stress proteins is accompanied by increased tumorigenicity in mouse tumor models [56–60]. Furthermore, increased hsp70 and hsp60 expression in ovarian and breast cancer, respectively, has been associated with an unfavorable prognosis [61, 62]. However, the increased tumorigenicity of tumors induced by increased stress protein expression and the immunogenicity of tumor stress proteins are not contradictory. A correlation between immunogenicity of tumors and hsp70 expression was reported in two mouse tumor models [63, 64].

IMPLICATIONS FOR CLINICAL STUDIES

The advantage of tumor stress protein vaccines over vaccines consisting of defined tumor antigens lies in the observations that (i) tumor stress protein preparations mirror the individual antigenicity of a tumor due to multiple and random mutations; (ii) stress proteins represent powerful adjuvants for the peptide antigens complexed to them; and (iii) tumor-derived stress proteins represent tumor rejection antigens *in vivo*. The latter has not been shown for most CTL-recognized shared tumor antigens, which currently are being investigated in vaccine trials [65, 66]. The multivalent nature of tumor stress protein vaccines might prevent escape mechanisms of the tumor by antigen loss, as described after vaccination with single defined peptide antigens [67]. The preclinical data on the efficacy of tumor stress protein vaccines led to ongoing clinical phase I studies of tumor-derived gp96 preparations as autologous tumor vaccines in renal cancer, melanoma (M.D. Anderson Cancer Center, Houston, TX), pancreatic cancer (Memorial Sloan–Kettering Cancer Center, New York, NY), and gastric cancer (University of Mainz, Germany).

These trials will answer the question of whether this tumor vaccine strategy is feasible for different tumor entities. The stable and enhanced expression of gp96 in human tumors, as described for colorectal cancer, represents an important precondition for the feasibility of autologous gp96 tumor vaccines in clinical trials. The yield of gp96

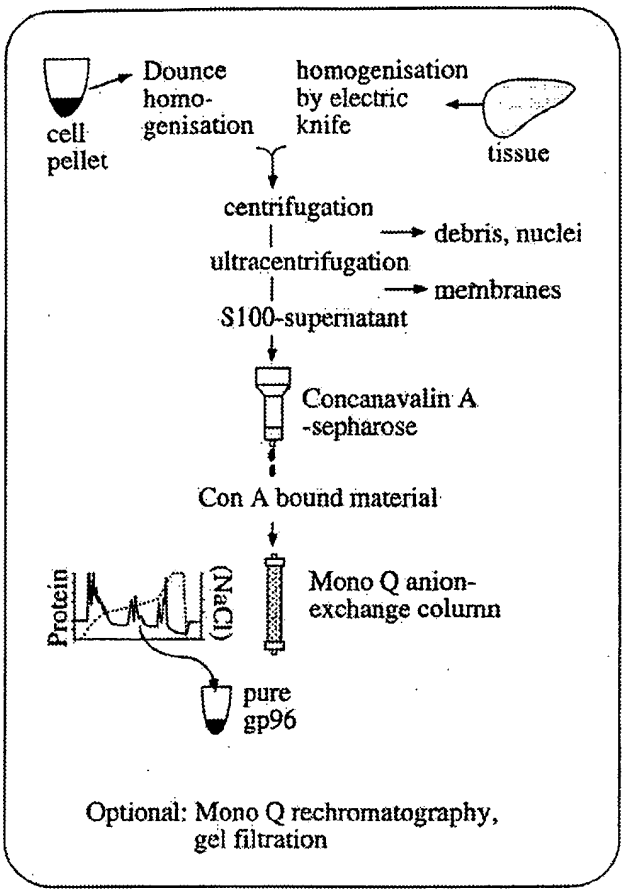

FIG. 2. Diagram of gp96 purification from cells or tissues by sequential column chromatography.

from tumor tissue is also very important for this vaccine approach. From melanoma cell cultures approximately 30 μg of gp96/g of cell pellet can be purified by a standard method described in Fig. 2 (Heike *et al.*, unpublished observations). Gp96 preparations from tumor tissue give yields of between 15 and 150 μg/g of tissue [68]. In murine tumor models, the induction of immunity by gp96 vaccination requires 10–20 μg of gp96 when injected subcutaneously two times one week apart, and only 1 μg of gp96 two times one week apart in the case of intradermal injection [68].

Another question of the trials will address the safety of stress protein vaccines. Since the endogenous tumor stress protein/peptide complexes certainly will contain peptides with normal sequences, the danger of causing an autoimmune reaction against these "self" epitopes is imminent. In mouse experiments no autoimmune reactions were observed [68]. However, this might be due to natural resistance of the investigated mouse strains against autoimmune diseases. In susceptible individuals thymic tolerance against "self" epitopes might not be complete, and peripheral tolerance might be broken by the strong adjuvant effect of stress proteins. Consequently, the clinical trials will need to monitor patients for autoimmune reactions carefully. In a

pilot trial, in which patients with end-stage tumor disease and different tumor entities were vaccinated with gp96 preparations derived from autologous tumor tissue, no autoimmune reactions or severe side-effects of the vaccine were observed [69].

A third question investigated by the clinical trials is whether the vaccination with autologous tumor-derived gp96 induces a T cell response in tumor patients against autologous tumor cells, or, in malignant melanoma, against known CTL-recognized tumor antigens. One endpoint of the trials is to find a vaccine dose that induces a T cell response, because this dose can be used in subsequent clinical efficacy studies. This endpoint is especially important in the gastric cancer and pancreatic cancer trials. In these trials, clinical efficacy cannot be assessed easily, because the trials include patients who underwent surgery with curative intent, although these patients have a high risk of relapse. In murine tumor models, the dose–response relation of gp96 vaccination can show a bell-shaped curve [33, 70]. It is not possible to extrapolate the efficient gp96 vaccine dose for humans from the mouse experiments. Consequently, a broad dose range has to be tested in the first trials of gp96 vaccination. The study of gp96 vaccination against pancreatic and gastric cancer in an adjuvant setting has the potential advantage that vaccination against tumors may work better with minimal residual disease than with a high tumor burden. A risk of these trials might be that the anti-tumor T cell reactivity induced by the vaccine might not reflect clinical efficacy. The induction of a T cell response against tumors is not necessarily accompanied by tumor regression in murine tumor models [71].

There are still a number of open questions concerning the mechanisms at work in stress protein vaccination. It is unclear how stress protein/peptide complexes are incorporated and processed by APC, how associated peptide antigens enter the MHC class I restricted antigen presentation pathway, and which APC are effective. It is also unclear how representative are the tumor antigens that are complexed with the respective stress proteins. It can be assumed that hsp70, hsp90, and gp96 are complexed with different repertoires of peptide antigens due to their specific subcellular locations and different binding properties. Peptide motifs of endogenous peptides complexed with the different stress proteins have not been published thus far. The answers to these questions will lead to further vaccine strategies. The problem of the bell-shaped dose–response curve for gp96 vaccination could be overcome by vaccination with gp96-pulsed professional APC-like dendritic cells, once they have been shown to process gp96/peptide complexes and present the associated peptides. Since cytoplasmic hsp70 and hsp90 are even more abundant than gp96 and possibly differ in their repertoire of associated immunogenic peptides, one can envision vaccinating patients with a panel of different stress proteins purified from autologous tumor tissue.

NOTE ADDED AT PROOF

Concerning the adjuvant function of stress proteins, a recent report described receptor-mediated endocytosis of stress proteins gp96 and hsp70 by murine APC (Arnold-Schild D, Hanau D, Spehner D, Schmid C, Rammensee H-G, de la Salle H, Schild H, Receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. *J Immunol* **162:** 3757–3760, 1999). Two additional phase I studies with tumor derived gp96 preparations as autologous tumor vaccines have started in melanoma and colorectal cancer (Istituto dei Tumori, Milan, Italy).

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1. Manjili MH, et al, Cancer Res 2002 Mar 15;62(6):1737-42
2. Srivastava, Annu. Rev. Immunol. 2002. 20:395-425.
3. Yedavelli SP, et al, Int J Mol Med 1999 Sep;4(3):243-8
4. Udono, H., et al, Int Immunol 13: 1233-1242
5. Hipp JD, et al, In Vivo 2000 Sep-Oct;14(5):571-85
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17. Silva CL. Microbes Infect 1999 May;1(6):429-35
18. Suzue K, et al, EXS 1996;77:451-65

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Chaperoning tumour antigens into the immune system

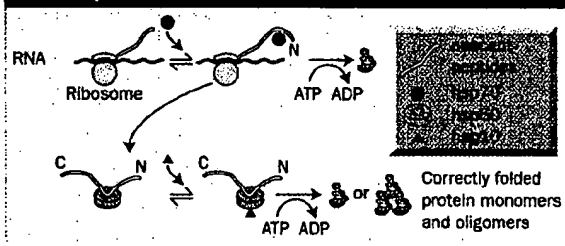
Many years of research have been spent trying to identify unique tumour antigens to use as therapeutic immunogens in cancer therapy. Recently, there have been some successes with this approach, most notably in the treatment of melanoma, but the wider picture looks less optimistic, mainly because it is difficult to find unique tumour antigens and to develop effective vaccines from them. A new approach where heat-shock proteins (hsps) are prepared from the patient's own cancer cells may offer a way to elicit an immune response against a broad and random sample of peptides from individual tumours, and may bring customised therapeutic cancer vaccines one step closer to reality.

Hsps are induced in cells that are under stress caused, for example, by ischaemia, fever, seizures, or haemodynamic overload. Research into hsps has given rise to potential clinical applications in a large number of areas, including wound healing, organ transplantation, and skin surgery. Hsps may be involved in some autoimmune diseases and, because their expression is characteristically elevated in some cancers, hsps may be useful diagnostic and prognostic markers.

From the cell's perspective, stress is any condition that results in the accumulation of unfolded or malfolded proteins. The cell responds by shutting down the synthesis of most proteins while dramatically increasing the expression of hsps. These rescue the cell by refolding unfolded proteins and by providing a degree of tolerance to subsequent abuse. But hsps are not only crisis managers. They are abundant and essential constituents of all cells, performing essential protein folding and refolding jobs (figure).

Pramod Srivastava (Fordham University, New York, USA) came across hsps while searching for immunogens by biochemically fractionating cancer cells and injecting the fractions back into rats with cancer. Srivastava purified a protein of about 100 kDa from both rat and mouse tumours that, when injected back into animals, caused rejection of the cancer from which it was purified. He recalls, "I was surprised to find what looked like the same protein [in both species], and then disappointed when we cloned and sequenced it to find it was an hsp". In fact, it turned out to be gp96, an endoplasmic reticulum form of hsp. Srivastava and his colleagues later found that cytoplasmic hsp70 could also generate immunity to the tumours from which it was derived.

What hsps do in unstressed cells



Hsp70 binds to nascent polypeptides as they emerge from the ribosome and facilitates correct folding. This may be sufficient to enable some proteins to fold correctly, but other proteins need the help of hsp60 and hsp10. Hsp70, hsp60, and hsp10 are cytosolic hsps. Other hsps are located in the endoplasmic reticulum and the mitochondria.
All hsps are molecular chaperones—ie, proteins that bind to non-native conformations of proteins in cells to assist their folding or refolding. Other chaperones include protein disulphide isomerase, peptidyl prolyl isomerase, calreticulin, calnexin, and nucleoplasmin.

So why are hsps purified from tumours apparently such good vaccines? Hsps bind to polypeptides and peptides almost irrespective of sequence, so when cancer cells are homogenised during protein purification, the hsps sample all the proteins in the cell including all the tumour-specific proteins. After purification, mainly peptides remain bound to the hsps because they have a higher affinity for hsps than do polypeptides. When injected into the bloodstream, the hsp–peptide complexes are taken up by professional antigen-presenting cells that then elicit protective T-cell responses.

"I have no idea what these cells do with the hsps", says Srivastava, "but the peptides they carry get presented by MHC molecules". Because the Hsp–peptide complexes are panvalent, "they chaperone the entire repertoire of tumour-cell proteins including cryptic or sub-dominant antigens", he explains, and this may mean that they work much better as vaccines than vaccines based on one or a small number of antigens.

For patients, Srivastava says his approach will have the advantage over other cancer vaccine approaches of customisation: each vaccine will have a unique set of antigens derived from the patient's own cancer. "This is not a one-shoe-fits-all approach", he says. "I believe that there is little evidence for the existence of shared antigens on tumours. Each tumour has a unique set of mutations and, therefore, a unique set of antigens."

Bill Welch, professor of medicine and physiology at the University of California at San Francisco, USA, and a founder of StressGen, a Canadian company developing hsp applications, finds Srivastava's data very intriguing, "but there's really not much evidence at the molecular level of what's going on", he comments. "Until the molecular details are filled in, I think we should still regard this as a curiosity, but it could be very exciting. If he's right, it is going to open up a lot of new avenues in antigen presentation".

A phase I trial has recently been completed by Srivastava and collaborators at the Hospital Charité in Berlin, Germany. As well as testing toxicity in 14 patients, the trial tested the logistics of the method: a tumour sample is taken from the patient during surgery or biopsy, the hsps are purified from the sample in the laboratory—a process that takes about 6 hours and that can be mechanised—and then injected back into the patient without the addition of adjuvants. The results of the first trial confirmed the feasibility of the approach and showed no toxicity. A second phase I trial is now planned in New York, USA, for patients with pancreatic cancer.

"Hsps are the first adjuvants of mammalian origin", claims Srivastava. He speculates that a system may have evolved to enable antigen presenting cells to scavenge hsps released from virally infected cells or from pathogenic bacteria. In this way, viral or bacterial peptides could be shunted directly into antigen-presentation pathways. Whether or not this hypothesis is correct, the hsp-mediated immunisation strategy may well be applicable to vaccination against intracellular infections as well as against cancer.

Carol Featherstone

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Heat shock protein–peptide complexes in cancer immunotherapy

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Heat shock proteins (HSPs) are associated with a broad spectrum of peptides derived from the cells from which they are isolated. Vaccination with such HSP–peptide complexes elicits protective immunity against tumors or other cells used as the source of HSPs. These observations suggest that HSP–peptide complexes are suitable as vaccines against cancers and infectious diseases.

Current Opinion in Immunology 1994, 6:728–732

Introduction

The use of HSP–peptide complexes for cancer immunotherapy is rooted deep in the history of cancer immunology. Immunogenic antigens of chemically induced tumors were first identified 50 years ago, and they still remain the most convincing examples of tumor-specific antigens [1]. By a combination of biochemical fractionation and tumor-rejection assays, these antigens were identified as HSPs [2–5]. A striking feature of tumor-rejection antigens of mice is their distinct nature: each tumor generally elicits immunity against itself but not against another tumor [1]. Tumor-derived HSPs also display this specificity and elicit immunity only against the tumors from which they are isolated [1–5]. In contrast to this functional specificity, genes encoding HSPs do not show tumor-specific DNA polymorphism [6]. In order to resolve this connundrum and in light of the ability of HSPs to bind to a diverse array of molecules [7], it was suggested that HSPs are not antigenic *per se*, but are carriers of immunogenic peptides [6,8]. The past year has seen the emergence of a number of observations that support this hypothesis. These observations and their implications for cancer immunotherapy are the focus of this article.

Role of HSPs in immune response to cancer

It will be instructive to place the present discussion in the larger context of the role of HSPs in immune response in general. HSPs continue to appear with intriguing regularity among antigens detected by immune response to infectious agents, cancers and autoantigens [9]. The apparent ubiquity of immunogenicity of HSPs has occasionally led to their comparison with superantigens, endotoxins, adjuvants and non-specific stimulators of immune response. In order to distinguish between the different mechanisms by which HSPs elicit and modulate a diverse array of immune responses, we have categorized the role of HSPs in immune response into four distinct paradigms [10].

In the first paradigm, seen in immunity to infection, HSPs of infectious agents act as classical foreign antigens, and elicit immunity because their sequences are different from the host's HSPs. The second paradigm includes instances where the host responds to self HSPs to which tolerance has not been established, or is broken. The third paradigm, which overlaps partially with the second, involves instances of molecular mimicry where cross-reactivity between an HSP and an unrelated protein leads to immune response to the latter under conditions which elicit immune responses to the former, such as infection with a bacterium whose dominant antigen is an HSP. The fourth paradigm, a major subject of this article, refers to observations where an HSP–antigen complex elicits immune response to the antigen and not to the HSP. The possible role of HSPs in recognition by γδ T cells may also fall into this paradigm.

Association of antigenic peptides with HSPs — a central tenet of the fourth paradigm

Apparently homogeneous preparations of the HSP gp96 derived from Meth A and mouse sarcomas have been shown to elicit tumor-specific immunity [3]. However, in view of lack of sequence differences in the gp96 genes among these tumors, it was suggested that the immunogenicity results from low molecular weight substances such as peptides, associated with gp96 and not from gp96 *per se*. In this regard, a key observation has recently been made [11••]: homogeneous gp96 preparations from

Abbreviations

CTL—cytotoxic T lymphocytes; **HSP**—heat shock protein; **MHC**—major histocompatibility complex.

 ISSN 0952-7915

metabolically labelled Meth A cells were treated with low pH under conditions used to elute peptides from major histocompatibility complex (MHC) molecules. The low molecular weight eluate was analysed by reverse phase chromatography, and a number of methionine labelled peptide peaks were detected. Preliminary characterization of these gp96 peptides has shown that they constitute a broad array of several hundred peptides derived from cellular proteins (PK Srivastava and H Udono, unpublished data). Interestingly, while these observations were made with gp96, which is an abundant HSP of the lumen of the endoplasmic reticulum (ER), similar results were obtained with a cytosolic HSP, HSP70 — the most extensively studied and arguably the most venerated HSP. HSP70 preparations from Meth A sarcoma elicited protection against Meth A, but not against the antigenically distinct CM4 or CMS5 sarcomas [12••]. The ability of HSP70 molecules to bind peptides *in vitro* and the lability of this interaction in the presence of ATP had already been demonstrated [7]. Hence, it was logical to treat the immunogenic hsp70 preparations of the Meth A sarcoma with ATP and test if the presumably peptide-depleted preparations were still antigenically active. ATP treatment did release a range of peptides from hsp70 and cause abrogation of immunogenicity of hsp70 [12••]. Collectively, these observations support the central tenet of the fourth paradigm.

The association of peptides with HSPs is not a tumor-specific phenomenon because it has also been observed in normal tissues. However, gp96 and hsp70 preparations derived from normal tissues were unable to elicit immunity to Meth A sarcoma in a range of doses tested [12••,13•], suggesting the presence of tumor-specific antigenic peptides in HSP preparations from tumors, but not in the preparations from normal tissues. Association of antigenic peptides with HSPs is also observed in virus-infected cells, and HSP–peptide complexes isolated from such cells do elicit antigen-specific cytotoxic T lymphocytes (CTLs) [14•].

Significant differences have been detected in the relative immunogenicity of the various HSPs [13•]. Immunogenicity of gp96, hsp90 and hsp70 preparations was tested by immunizing mice with different doses of each HSP–peptide complex followed by different levels of challenge of the vaccinated mice with live tumor cells. Two key results were obtained: gp96 and hsp70 have almost 10 times more specific immunogenicity than hsp90; and a threshold dose of HSPs is necessary to protect even against lower tumor challenges. Smaller doses of vaccination do not necessarily protect against smaller tumor challenges. The cellular basis of these observations is not yet clear.

Recognition of tumor cells by γδ T cells may also fall under the fourth paradigm. Although the ligands for γδ-receptors have not been identified, there is a tantalizing connection between them and the HSPs [15]. During the search for the structural basis of specific antigenicity of tumor-derived HSPs, it was earlier proposed [6] that HSPs directly present peptides to T cells, in the same manner as MHC molecules. However, the model was considered unlikely as specific T-cell recognition of tumor cells was blocked by anti-MHC class I antibodies but not by anti-gp96 antibodies. Recent observations appear to have caught up with the essential logic of that early model. Tamura *et al.* [16••] have shown that cytotoxicity of a double-negative T-cell line against a tumor cell depends on the expression of a cell surface hsp70 by the tumor cell and can be blocked by an anti-hsp70 antibody, but not by anti-MHC class I or anti-NK antibodies. Similarly, Kim *et al.* (A. Krensky, personal communication) have demonstrated that cytotoxicity of human γδ T cells against an autologous B-cell lymphoma is blocked by anti-HSP75 but not by anti-MHC class I antibodies. In this system, recognition by γδ T cells is antigen-specific and the antigenic moiety has been structurally characterized. Further, recognition of the target cell by the γδ-cells requires antigen processing in the B-cell lymphoma. In this latter respect, these studies are distinct from those of Sciammas *et al.* [17], who show that unprocessed antigen can be recognized by an antigen-specific γδ T cell clone. The demonstration of cell surface expression of a number of HSPs ([3,6,16••]; G Multhoff *et al.*, abstract 330, Cold Spring Harbor, New York, May 1994; A Altmeyer, *et al.*, unpublished data) is consistent with the possibility of HSPs acting as presenting molecules to γδ cells. It is conceivable that similar to the situation with αβ T cells, such recognition may be antigen-specific in instances where a specific peptide is recognized in context of HSP molecules; in other instances, comparable to allogeneic recognition by αβ T cells, the nature of the peptides may be less germane.

Structural characterization of peptides associated with gp96, hsp90, hsp70 and other HSPs with respect to their size, sequence and heterogeneity is an obvious next step in further elucidation of the fourth paradigm. These studies have obvious implications for antigen processing and presentation of antigenic peptides by MHC molecules. However, these aspects are beyond the scope of this article and are discussed elsewhere [18,19••].

It is important to distinguish studies on the use of HSP–peptide complexes in vaccination from apparently similar studies where a foreign HSP transfected into a tumor renders the tumor highly immunogenic [20]. In the latter, the HSP is an antigen in its own right by virtue of being bacterial and foreign. Transfection of a murine tumor with any bacterial or other foreign gene (not only an HSP gene) will render murine tumors immunogenic in mice. The classical observation that tumor cells transfected with the foreign HSP gene also elicit resistance to the untransfected tumors has been made previously in a number of systems [21] and is unrelated to the transfected gene being an HSP.

The essential role of macrophages in immunity elicited by HSP–peptide complexes

Examination of the cellular mechanism through which HSP–peptide complexes elicit protective tumor immunity has begun. Mice have been depleted of specific sub-sets of T cells or phagocytic cells during the priming or effector phases of HSP–peptide elicited tumor immunity and the consequence of such depletion monitored [22••]. Depletion of $CD8^+$, but not $CD4^+$ T cells in the priming phase abrogates immunity elicited by gp96. Depletion of functional macrophage by treatment of mice with carrageenan during the priming phase also results in loss of gp96-elicited immunity. In the effector phase, all three compartments, $CD4^+$ and $CD8^+$ T cells and macrophages, are required. Surprisingly, immunity elicited by irradiated tumor cells shows different requirements. In contrast to immunization with gp96, depletion of $CD4^+$, but not $CD8^+$, T cells during priming with whole tumor cells, abrogates immunity. Ablation of macrophage during priming or effector phases has no effect on immunity elicited by whole cells. These results indicate an essential and distinct role for macrophage in T-cell immunity elicited by HSP–peptide complexes.

The experiments described above suggest that the high specific immunogenicity of HSP–peptide complexes may derive from their ability to be taken up by the macrophage using a specific mechanism, such as a receptor, which may channel the HSP-chaperoned peptides into the endogenous presentation pathway of the macrophage [19••]. This will result in re-presentation of the peptide in the context of the MHC class I of the macrophage. The presence of co-stimulating molecules, such as B7, on the macrophage may make the presentation of peptide by macrophage MHC class I extremely productive for a CTL response. The role of macrophages or similar cells in channeling exogenous antigens to the endogenous pathway has also been suggested independently [23,24].

HSP–peptide complexes as therapeutic vaccines against cancer

In view of the specific antigenicity of chemically induced mouse tumors [1], it has long been expected that human tumors also show antigenic individuality. If this turns out to be true, then prospect of developing a general or even a quasi-general vaccine against human cancers would be impractical. In this context, the ability of tumor-derived HSP–peptide complexes to elicit tumor-specific immunity points to a practical solution: HSP preparations derived from surgically resected tumors can be used to vaccinate patients, without requiring prior knowledge of the antigenic epitopes of a particular tumor. Thus, although the T-cell epitopes of Meth A, CMS4, CMS5 or other mouse tumors are unknown, it is possible to vaccinate against them effectively, by using HSP–peptide complexes derived from them. Further, as a number of HSPs can now be purified rapidly, a means of preparing customized, patient-specific cancer vaccines is within reach.

However, the idea that human tumors, like their murine counterparts, are antigenically distinct, is presently being contested [25]. Identification of the CTL epitopes from a number of human melanomas as shared, unmutated differentiation antigens has kindled the hope that human tumor antigens are cross-reactive, and that customized, patient-specific vaccines will not be necessary. These results also pose an intriguing question. Extensive characterization of autologous antibody response to human melanomas previously led to identification of differentiation antigens and other shared antigens as tumor antigens [26,27]. The new results obtained with CTLs of melanoma patients appear to be qualitatively comparable to the results obtained with sera of melanoma patients. In that context, are the CTLs from melanoma patients 'scanning' the antigenic repertoire of tumor cells, just as the antibodies of an earlier era appeared to do, or are the results qualitatively new and more relevant for cancer immunotherapy? It may also be instructive to recall that shared antigens can be readily detected even on chemically induced mouse sarcomas [1,28] and T-cell response against such shared antigens can be generated [29•]; however, the shared antigens are may be weak and do not elicit significant transplantation immunity. Solving these dilemmas will be crucial for considering the use of shared antigens for immunotherapy of human melanomas.

In the happy event that sets of immunoprotective shared antigens are identified for tumors of different lineages, vaccination of patients with peptides or peptide-based constructs derived from the appropriate differentiation antigens would appear to be a method of choice. In spite of the obvious appeal of this approach, it raises a number of logistical questions. Clearly, vaccines will have to contain epitopes derived from a number of antigens. For any particular antigen, vaccination with a given peptide will be effective only for patients with a given HLA allele. If different epitopes from a single molecule such as tyrosinase, are recognized by different HLA alleles [25], a cocktail of peptides will have to be used for vaccinating a general population. Even for a given patient, a cocktail may have to be used, as humans are outbred and possess several restriction elements. In the light of this, peptide-based vaccines may have to be inordinately large chimaeric constructs. Isolation of HSP–peptide complexes from allogeneic human lines expressing the appropriate antigens could be a more practical alternative. HSPs are non-polymorphic and their association with peptides is proximal to the association of MHC class I with peptides in the antigen presentation pathway [19••]. Thus, peptides associated with an HSP are not selected for the HLA specificity and represent a collection of epitopes (or epitope-precursors) corresponding to all possible HLA specificities. Vaccination with such

complexes derived from an allogenic tumor will also immunize patients regardless of their HLA haplotype. Thus, it was predicted that HSP–peptide complexes can be effective in cross-priming across the HLA barrier, and that matching a patient's HLA phenotype with that of a tumor vaccine is unnecessary [19••]. This last idea has also been recently derived from another angle [30•]. It would appear therefore that regardless of whether human tumor antigens turn out to be individually specific, cross-reactive, or both, vaccination with HSP–peptide complexes provides a means of circumventing many of the hurdles associated with other peptide-based approaches.

The inherently multivalent nature of HSP–peptide complexes is an added advantage in vaccination. Individual human cancers are heterogeneous with respect to most parameters and antigenicity is unlikely to be an exception. Vaccination of patients with one or few peptides is expected to lead to selective elimination of tumors and outgrowth of immunological escape variants. Vaccination with HSP–peptide complexes makes immunological escape virtually impossible because the vaccines contain not one or a few peptides but the entire antigenic repertoire of that tumor.

Finally, vaccination with HSP–peptide complexes elicits long-lasting T-cell immunity [12••]. Recent results show that antigen-specific T cells elicited by vaccination with HSP–peptide complexes are radiation-resistant and display characteristics of memory T cells (S Janetzki, NE Blachere and PK Srivastava, unpublished data). This attribute of HSP–peptide complexes fulfills a crucial requirement for a suitable vaccine.

Future research

Although the central tenets for the use of HSP–peptide complexes in immunotherapy have been substantially strengthened during the past year, a number of things remain to be done. These include expansion of the fourth paradigm to other tumor models, demonstration of its efficacy in therapeutic as opposed to prophylactic vaccination, elucidation of further the cellular mechanism by which HSP–peptide complexes elicit protective immunity, determination of the optimal dosage and frequency of administration of HSP–peptide complexes in patients, and exploration of the use of adjuvants with HSP–peptide complexes. Progress in these areas is expected to result in translation of the fourth paradigm into a new generation of vaccines against cancers and intracellular infectious agents. It should also lead to a resolution of the structural basis of specific antigenicity of methylcholanthrene-induced sarcomas of inbred mice — an enigma associated with the beginning of time in cancer immunology.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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Heat shock protein-peptide complexes for use in vaccines

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Abstract: The heat shock proteins gp96, HSP70, and HSP90 are complexed to a diverse array of cellular proteins and peptides as a consequence of their chaperone functions. There is good experimental evidence that vaccination with these heat shock protein-peptide complexes elicit immune responses against chaperoned peptide antigens. As shown with gp96, this requires internalization of the heat shock protein-peptide complexes by macrophages and processing of the chaperoned peptides for class I restricted presentation. Via this process, primarily $CD8^+$ antigen-specific T cells are primed by gp96 vaccination. This might represent a general mechanism for priming of MHC-class I restricted T cells by professional antigen-presenting cells. At least gp96 has been shown to be associated with peptides that are not selected by the MHC haplotype of the harboring cell. This allows for immunization with gp96-peptide complexes across MHC barriers, for example against shared tumor antigens or viral antigens; *J. Leukoc. Biol.* 60: 153–158; 1996.

Key Words: *gp96 · HSP70 · HSP90 · T cells · tumor immunology · antigen presentation*

INTRODUCTION

The idea that heat shock protein-peptide complexes could serve as vaccines stems from the identification of members of the HSP90 family, gp96 in the endoplasmic reticulum and cytosolic HSP90 molecules, and of the HSP70 family as tumor rejection antigens of chemically induced mouse tumors [1–5]. These heat shock proteins elicit a strictly tumor-specific transplantation immunity against the tumor they had been isolated from, but not against antigenically distinct tumors [1, 2, 5]. Heat shock proteins isolated from normal tissues do not elicit tumor immunity [5–7]. Heat shock protein genes do not show tumor-associated polymorphism [4, 8–10], which could have explained their diverse immunogenicity. Therefore, it was assumed that heat shock proteins elicit immunity in the vaccinated organism by presenting immunogenic peptides from the cells of origin [11]. In this way, the immunogenicity of heat shock proteins would reflect the antigenicity of the harboring cells. This concept fits well in the role of heat shock proteins as cellular chaperones, which bind to a diverse array of unfolded or partially folded proteins in every cellular compartment [12–14]. In the following, we review: 1) the evidence for the concept that vaccination with heat shock proteins gp96, HSP70, and HSP90 elicits antigen-specific immunity by bound immunogenic peptides, and 2) the cellular mechanisms involved in the immunogenicity of these heat shock proteins. We start with gp96 because the immunogenicity of this molecule and the mechanisms involved have been investigated most extensively.

GP96, AN ENDOPLASMIC CHAPERONE FOR IMMUNOGENIC PEPTIDES?

It is most likely that gp96 is identical to ERp99 [15] and the glucose-regulated protein GRP94 [16] on the basis of DNA sequence homology [4]. For reasons of simplicity, we refer to both of these molecules as gp96. gp96 is among the most abundant proteins in the endoplasmic reticulum and is retained there because of a KDEL signal peptide. It is related to the HSP90 family of cytosolic heat shock proteins, sharing 50% homology with them [4]. gp96 is expressed constitutively but can be up-regulated by the presence of malfolded proteins in the endoplasmic reticulum (ER) [17], which resulted from mutations or various stress factors such as heat shock [4, M. Heike, B. Noll, W. Dippold, U. Schmitt, P.K. Srinivastava, and K.-H. Meyer zum Büschenfelde, unpublished results], disturbance of glycosylation [16], and pertubation of intracellular calcium balance [18].

Chaperone functions of gp96

The chaperone functions of gp96 are central in binding immunogenic peptides. gp96 acts as a chaperone in concert with GRP78, an ER resident protein of the HSP70 family. Like HSP70, gp96 has ATPase activity [19], possibly necessary for the exchange of bound proteins or peptides. Both ER resident stress proteins, gp96 and GRP78, retain and stabilize assembly intermediates of macro-

Abbreviations: ER, endoplasmic reticulum; VSV, vesicular stomatitis virus.

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Received February 22, 1996; accepted February 22, 1996.

molecular proteins in the ER before they reach a transport-competent state and can be secreted. In addition, they inhibit the secretion of terminally malfolded proteins. These functions are shown by the following examples: Gp96 has been found together with GRP78 in stable aggregated complexes with aberrant viral proteins in the ER [20], and gp96, ERp72, another abundant ER protein, and GRP78 were found in complexes with MHC class II chains in the ER, which are formed in the absence of the invariant chain [21, 22]. Most likely these proteins transiently retain and stabilize class II chains in the ER during assembly of the transport-competent complex between class II chains and invariant chains. gp96 was also shown to associate with free immunoglobulin chains before their assembly into active dimers [23, 24].

Binding specificity of gp96

What is known about the binding specificity of gp96 or the structural requirements of proteins or peptides that bind to gp96? GP96 has been demonstrated to bind to more mature, fully oxidized immunoglobulin chains than GRP78 is able to bind [24, 25]. This could be explained by the different ATPase activity of gp96 compared with GRP78. The gp96 ATPase is not stimulated by peptides like the ATPase activity of GRP78, but is stimulated by partially unfolded proteins like casein [19]. If ATP hydrolysis is necessary for the binding of gp96 to its substrate, gp96 can be expected to bind to more mature protein structures than GRP78. However, gp96 also binds peptides. A diverse array of several hundred cellular peptides between 400 and 2000 kDa have been found to be associated with gp96 molecules in an approximate ratio of 1:1 [19, 26]. These peptides were tightly bound to gp96, not dissociating at salt concentrations as high as 0.5 M NaCl. They could be eluted by acid extraction as reported for MHC-class I bound peptides. In addition to the association with non-viral cellular peptides, gp96 associated with peptides from the vesicular stomatitis virus (VSV) nucleocapsid protein, a major target for VSV-specific CTL [27].

These findings show that gp96 can bind immunogenic cellular peptides with relative promiscuity. It was also reported that gp96 could be co-immunoprecipitated with MHC-class I [19]. These findings led to a model in which gp96 plays a major role in MHC-class I restricted antigen presentation [19, 28]. It was proposed that gp96 is charged with peptides from TAP transporters, which are responsible for the transport of immunogenic peptides into the ER. The interaction between gp96 and MHC class I activates the gp96 ATPase. ATP hydrolysis generates energy to transfer peptides from gp96 to MHC class I. It is not known, so far, if activation of the gp96 ATPase actually releases bound peptides.

Immunogenicity of gp96

What is the evidence for the concept that bound peptides are responsible for the immunogenicity of gp96? gp96 isolated from chemically induced mouse sarcomas has been shown to elicit tumor-specific transplantation immunity against the tumor from which it was prepared but not against antigenically distinct syngeneic tumors [1]. gp96 isolated from normal tissue did not elicit transplantation immunity against tumors [6, 7]. Immunization of mice with gp96 isolated from antigenically different cells elicited an antigen-specific CTL response. This could be shown for influenza nucleoprotein-transfected cells, SV40-transformed cells, UV-induced sarcoma cells [6], VSV-infected cells [27], β-galactosidase-transfected and minor H antigen-bearing cells [29]. The latter study showed that gp96 isolated from a cell line with two known target antigens for CTL, β-galactosidase, and H-minor antigens, could elicit a CTL response against both antigens. This confirmed that gp96 from one cell may confer immunity against different cellular antigens. These studies show that the immunogenicity of gp96 from a given cell reflects the particular antigenicity of this cell. These observations and the chaperone function of gp96 described above lead to the conclusion that the cellular peptides chaperoned by gp96 are responsible for the antigen-specific immune response elicited by gp96 preparations.

Cross-priming by gp96

Is the immune response elicited by gp96-peptide complexes dependent on the MHC-type of the cellular source of gp96? Peptides bound by gp96 should represent a sample of cellular protein epitopes of yet unknown diversity and should not be influenced by the MHC haplotype of the cell from which gp96 was isolated. Therefore, gp96 should elicit immune responses against cellular antigens in all responders irrespective of MHC-haplotypes. This phenomenon is called cross-priming and has already been observed for immune responses elicited by whole cells: cells expressing a certain antigen could prime antigen-specific $CD8^+$ T cells across MHC barriers [30, 31]. Two recent studies showed that gp96 can prime $CD8^+$ T cell responses across MHC barriers. Arnold et al. [29] showed that gp96 from a murine cell line with an $H\text{-}2^d$ background and multiple minor H antigens could prime $CD8^+$ T cells of $H\text{-}2^b$ mice against these minor H antigens. Suto and Srivastava showed that gp96 isolated from VSV-infected cells of $H\text{-}2^d$ haplotype elicited a VSV-specific CTL response in mice of the $H\text{-}2^b$ haplotype [27]. These experiments show that peptides chaperoned by gp96 represent epitopes not selected by the MHC haplotype of the harboring cell.

Cellular mechanisms involved in the immunogenicity of gp96

The tumor-specific immunity elicited by gp96 could be adoptively transferred by T cells from immunized mice to syngeneic naive mice [32]. In case of UV-induced sarcomas it could be shown that immunization with gp96 elicits a tumor-specific, radiation-resistant, $CD8^+$ memory T cell response [S. Janetzki, N.E. Blachere, and P.K. Srivastava, unpublished results]. Cellular requirements for a success-

ful immunization with gp96 were tested by in vivo depletion of mice for $CD4^+$ or $CD8^+$ T cells or macrophages during the priming and effector phases of the immune response [33]. It could be shown that immunization with gp96 elicits a $CD8^+$ but not a $CD4^+$ T cell response. This in vivo priming of $CD8^+$ T cells by gp96 did not occur if the mice were treated with carageenan before immunization. Because carageenan treatment abrogates macrophage function, it was hypothesized that macrophages internalize and process gp96, and hence channel immunogenic peptides, which have been chaperoned by gp96, into the MHC class I restricted antigen-presentation pathway. This concept was confirmed by a recent study which demonstrated that gp96-bound VSV antigens could be processed in vitro by macrophages for MHC class I presentation [27]. This was shown with two different gp96 preparations: 1) gp96 isolated from VSV-infected cells and 2) in vitro reconstituted gp96-VSV peptide complexes. Pretreatment of macrophages with BFA abrogated the processing of gp96, whereas chloroquin had no effect. This indicates that gp96-peptide complexes are indeed internalized by macrophages and are processed in non-acidic compartments. Remarkably, sensitizing macrophages against CTL with gp96-peptide complexes required a quantity of peptide several hundred times lower than the amount required when pulsing with free peptides.

HSP70, UBIQUITOUS CELLULAR CHAPERONES FOR IMMUNOGENIC PEPTIDES?

The HSP70 family is a multigene family. Members have been found in every cell compartment of eukaryotic cells. In the cytosol HSP73 is constitutively expressed, whereas HSP72 is heat inducible. Both proteins share 90% homology [12, 13]. PBP72/74, a member of the HSP70 family, is located in endosomes and plasma membranes, and is involved in class II restricted antigen presentation [34–37]. GRP75 is located in the mitochondrial matrix. In the ER GRP78 is one of the abundantly expressed stress proteins along with gp96. Expression of both stress proteins is transcriptionally up-regulated by the accumulation of malfolded proteins in the ER [17]. Both stress proteins, GRP78 and gp96, are the major ATP binding proteins in the ER [38] and possess ATPase activity.

Chaperone functions of HSP70

HSP70 family members have two functional domains: a highly conserved N-terminal domain (44 kDa), which contains a nucleotide binding site, and a divergent C-terminal domain, which is involved in binding to unfolded proteins or peptides [13]. Binding to ATP in the presence of K^+ causes a conformational change of HSP70, leading to protein or peptide dissociation, whereas ATP hydrolysis increases affinity of HSP70 for peptides [14, 39]. Consequently, the exchange of bound proteins or peptides by HSP70 is catalyzed by cycles of ATP binding and ATP hydrolysis. Numerous functions have been ascribed to HSP70 proteins [14]: binding to nascent polypeptide chains on ribosomes to prohibit early malfolding [40], maintaining translocation-competent states of proteins destined for the ER and mitochondria [41], blocking of non-productive folding interactions of partially unfolded proteins during stress [42, 43], retaining of assembly intermediates and supporting assembly of membrane proteins like immunoglobulin or MHC-class II in the ER [21, 25], and retaining terminally malfolded proteins in the ER [20, 44, 45]. Generally, HSP70 molecules bind transiently to folding intermediates but stably to proteins malfolded due to mutations or stress.

Binding specificity of HSP70

HSP70 sequences could be aligned to the peptide binding domain of HLA-class I, suggesting a peptide binding groove for HSP70 similar to HLA-class I [46]. NMR studies have shown that peptides composed of 13 residues are bound in an extended conformation to DnaK, the *Escherichia coli* homologue to HSP70 [47]. Binding studies with random peptides showed that short peptides with hydrophobic or with alternating aromatic and hydrophobic amino acids and seven to eight residues are bound preferentially by GRP78 [48, 49]. Stretches of amino acids, which would bind to GRP78, were estimated to occur every 16 residues in a globular protein [48]. Together, these results show a relatively promiscous binding of HSP70 to non-native proteins or peptides. With regard to binding of immunogenic peptides in neoplastic and infected cells, HSP70 molecules have been shown to be complexed with oncogenic proteins like mutant p53 [50, 51], v-rel [52], and numerous viral membrane proteins, such as VSV-G-protein [44], HSV-1 glycoprotein B [20], and vaccinia proteins [53]. It can be expected that in viral infections, which suppress the transcription of cellular proteins with the exception of HSP70 and HSP90 proteins, HSP70 will bind preferentially viral epitopes [53]. In tumor cells, the ratio of malfolded proteins bound stably to HSP70 may be higher than in normal cells due to increased mutation rates in these cells as a consequence of the carcinogenic influence and inherent DNA replication errors. HSP70-peptide complexes carrying these mutated epitopes are potentially useful in eliciting a tumor-specific immune response.

Immunogenicity of HSP70

There are now several reports which indicate that HSP70 molecules associate with immunogenic peptides in cells and play a role in antigen presentation to T cells. PBP72/74, a member of the HSP70 family was identified by binding to a cytochrome *c* peptide recognized by helper T cells. Antisera against PBP72/74 could block presentation of the cytochrome *c* peptide to an antigen-specific T cell hybridoma [34, 35]. PBP 72/74 is located in endosomes and plasma membranes, where it is possibly involved in peptide binding to MHC-class II molecules [36, 54]. Another recent study described the possible involve-

ment of HSP70 in class I restricted antigen presentation by a TAP transporter-independent pathway [55]. Two reports have shown that HSP70 can act as an antigen-presenting molecule on tumor cells. In one report the recognition of a processed immunoglobulin λ-chain on human lymphoma cells by autologous γδ CTL could be blocked by antibodies against the heat shock protein grp75 [56]. In the other report the recognition of an H-ras oncogene-transformed rat fibrosarcoma by $CD4^-CD8^-$ double-negative T cells could be blocked by antibodies against a surface HSP70 molecule [57]. Favorable for the use of HSP70-peptide complexes in vaccines is the potential adjuvant effect of HSP70. It was shown that vaccination with malaria peptide antigens or meningococcal oligosaccharides that were bound to HSP70 elicited high specific antibody titers without adjuvants. This adjuvant effect of HSP70 was T cell dependent [58]. Another example of the adjuvant effect of HSP70 is antibody response against p53 in cancer patients, which is only elicited if mutated p53 is complexed to HSP70 in tumor cells [59]. The adjuvant effect of HSP70 may also explain the increased immunogenicity of tumor cell variants expressing inducible HSP70 in an experimental tumor model [60]. The strongest support for the concept that HSP70-peptide complexes could be used as vaccines came from recent reports that vaccination with HSP70-peptide complexes isolated from tumor cells elicited a tumor-specific transplantation immunity, as it had been demonstrated for gp96-peptide complexes [5, 7]. Tumor-derived HSP70, which had been treated with ATP for peptide dissociation, did not elicit this immune response. From this it can be concluded that peptides bound to HSP70 are responsible for the tumor-specific immunity.

HSP90, CYTOSOLIC CHAPERONES FOR IMMUNOGENIC PEPTIDES?

Chaperone function of HSP90

HSP90 proteins are highly abundant and essential under normal conditions, making up 1–2% of cytoplasmic proteins in eukaryotic cells. Heat shock induces their expression several-fold. These proteins are members of a multigene family [9] with molecular masses between 82 and 90 kDa. The substrate specificity of HSP90 seems to be more restricted than that of HSP70. In most cases HSP90 binds to proteins involved in signal transduction such as steroid receptors and kinases in their near native form [13, 14]. Binding of HSP90 to these targets has a regulatory role. For example, HSP90 binding to the glucocorticoid receptor keeps the receptor in a hormone-binding but DNA-non-binding conformation [61]. HSP90 has also been shown to act as a chaperone in preventing the aggregation of unfolded proteins [62]. The specificity of HSP90-protein interaction or structural requirements of the substrates are still unknown. HSP90 possess ATPase activity [63] where ATP can induce the dissociation of bound proteins from HSP90 [64]. This suggests that the exchange of bound substrates by HSP90 is ATP dependent as it was shown for HSP70.

Immunogenicity of HSP90

Cytosolic HSP90 proteins have been identified as specific tumor rejection antigens of the chemically induced mouse sarcoma Meth A independently from the identification of gp96 in the same role [2, 3]. As shown for gp96 and HSP70, no cross-protection against antigenically distinct tumors was elicited by HSP90. Remarkably, related proteins were identified as tumor rejection antigens of SV40-induced tumors with no cross-immunity to Meth A [2]. The role of HSP90 as a tumor-specific transplantation antigen was confirmed recently [7]. However, the immunogenicity of HSP90 was inferior to that of HSP70 and gp96 on a quantitative basis.

CONCLUSIONS AND FUTURE RESEARCH

The stress proteins gp96, HSP70, and HSP90 are complexed to various cellular proteins and peptides as a consequence of their chaperone functions. The binding specificities of these stress proteins differ, with obviously broad specificities of gp96 and HSP70 and a more restricted specificity for HSP90. There is good experimental evidence that vaccination with these heat shock protein-peptide complexes elicits immune responses against chaperoned peptide antigens. As shown for gp96, this requires internalization of the heat shock protein-peptide complexes by macrophages and processing of the chaperoned peptides for class I-restricted presentation. Via this process, primarily $CD8^+$ antigen-specific T cells are primed by gp96 vaccination. It has been suggested that this represents a general mechanism for priming of MHC-class I-restricted T cells by professional antigen-presenting cells [65]. At least gp96 has been shown to be associated with peptides that are not selected by the MHC haplotype of the harboring cell. This allows immunization with gp96-peptide complexes across MHC barriers, for example against shared tumor [66, 67] or viral antigens. This mechanism would also explain the classical phenomenon of cross-priming of antigen-specific T cells by cells expressing the antigen but differing in the MHC haplotype.

The evidence that gp96, HSP70, and HSP90 bind immunogenic peptides led to the proposal of a relay line model for peptide transfer during antigen processing and presentation [28]: cytosolic HSP70 and HSP90 accept peptides, which were generated by proteases, most likely proteasomes [68], and transfer them to TAP peptide transporters spanning across the ER membrane. These transporters transfer the peptides to gp96 in the ER lumen. Gp96 finally transfers peptides to the MHC-class I heavy chain/β2m complexes. During this transport, peptides are gradually trimmed to the optimal length required for binding to MHC-class I. The peptide exchange between the members of this relay line is driven by the ATPase activities of the heat shock proteins. Associations of connected

members in this hypothetical relay line have so far been shown only for gp96 and MHC-class I by co-immunoprecipitation [19]. It should be noted, however, that GRP78 was also found to be complexed to class I heavy chains and that class I heavy chain/β2m complexes associate with the TAP transporter directly [22]. A modification of the above model might therefore be that TAP transporters directly transfer peptides of optimal size and motifs to MHC-class I molecules. Other peptides, which do not have the motif required for MHC class I binding, are primarily bound by GRP78 and gp96 in the ER and transferred to MHC-class I if the motif has been generated by gradually trimming. If and how HSP-associated peptides are trimmed in the ER remains unclear.

If gp96, HSP70, and HSP90 have an essential function in antigen processing, disturbances of this function in tumor cells would probably represent an additional immune escape mechanism along with HLA-class I losses or TAP transporter defects already demonstrated in tumor cells. However, heat shock proteins might also confer growth advantages for tumor cells [69]. Several reports show an increased expression of HSP70 and HSP90 in different human tumors [70–72] and an aberrant expression on the cell surface [73].

The heat shock proteins reviewed here appear to bind to a diverse sample of the antigenic repertoire of a cell. Aberrant malfolded proteins destined for degradation are possibly the prefered source of chaperoned peptides. In this case, epitopes from viral antigens or mutated proteins predestinated for malfolding would predominate over epitopes from normal proteins in binding to heat shock proteins. This would increase the efficacy and specificity of an immune response elicited by heat shock protein-peptide complexes against viral or tumor antigens. However, it cannot be concluded from the current experimental evidence that these heat shock proteins represent the entire antigenic repertoire of a cell or bind to immunodominant epitopes of every tumor or infection. The efficacy of these heat shock proteins as vaccines must be tested in other tumor models and models of viral infections with defined T cell antigens, namely in human tumor models. It would be of great interest to know whether gp96, HSP70, or HSP90 peptide complexes isolated from one cellular source contain peptides that represent different immunogenic epitopes. This for example, could be tested with gp96 from human melanomas. For these tumors, several CTL-defined antigens have recently been identified [66, 67], and one melanoma usually presents several CTL-defined antigens [74, 75].

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Thank you.

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Article title	The potential use of heat-shock proteins to vaccinate against mycobacterial infections
Article identifier	1286457999004864
Authors	Silva_C_L
Journal title	Microbes and Infection
ISSN	1286-4579
Publisher	Elsevier France
Year of publication	1999
Volume	1
Issue	6
Supplement	0
Page range	429-435
Number of pages	7
User name	Adonis
Cost centre	
PCC	$22.00
Date and time	Friday, June 14, 2002 3:16:51 AM

Microbes and Infection, 1, 1999, 429-435

Review

The potential use of heat-shock proteins to vaccinate against mycobacterial infections

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ABSTRACT – Over the last few years, some of our experiments in which mycobacterial heat-shock protein (HSP) antigens were presented to the immune system as if they were viral antigens have had a significant impact on our understanding of protective immunity against tuberculosis. They have also markedly enhanced the prospects for new vaccines. We now know that the mycobacterial HSP65 antigen can confer protection equal to that from live BCG vaccine in mice. © Elsevier, Paris

heat shock proteins / vaccine / tuberculosis / *Mycobacterium tuberculosis* / public health / control

1. Introduction

Heat-shock proteins (HSPs) belong to a group of proteins encoded mostly by intronless genes ubiquitously induced under stress conditions in all prokaryotic and eukaryotic cells. Because of their function, e.g., involvement in correct folding of proteins or their transport through intracellular membranes, they have also been named chaperonins. They display a striking structural homology, as evidenced by conserved DNA and protein sequences throughout evolution [1].

Evidence is accumulating that HSPs are major antigens of many pathogens [2]. As stated previously, the stress imposed by the host on its predators may lead to increased HSP synthesis. Due to their abundance, HSPs become prominent antigens that trigger a major portion of the immune repertoire. Members of the HSP70 and HSP60 families represent major targets for antibodies in many infections with helminths, protozoa, and bacteria [3]. T cells are also activated against HSP65 [1, 3–5]. Thus, in tuberculosis and leprosy patients, T cells with specificity for HSP65 have been repeatedly identified. In mice immunized with *Mycobacterium tuberculosis*, 10 to 20% of all T cells which respond to *M. tuberculosis* are specific for HSP65, as assessed by limiting dilution analysis [5]. Interestingly, T cells with reactivity to HSP65 have also been identified in normal healthy individuals lacking any clinical signs of disease [6]. These findings indicate that the immune response to HSP65 is often directed against epitopes shared by various microbes. As a corollary, the identification of antibodies or T cells against such shared regions cannot be taken as indicative of infection with a particular pathogen. Previous subclinical infections with a variety of microorganisms could have induced a certain level of immunity of apparently nonspecific character, but in fact specific for a shared epitope [2].

Recent years have seen the emergence of compelling evidence that HSPs possess unique properties that permit their use in generating specific immune responses against cancers and infectious agents; these applications suggest a critical role for HSPs in adaptive and innate immunity in vivo and raise the possibility that HSPs are the primordial molecules involved in host defense [7]. Although it is not clear whether this type of immunity contributes to protection against microbial pathogens, it has been suggested that it adds to resistance at an early stage of infection. Thus, cross-reactive immunity to HSPs of different microbes may fill the gap between natural resistance and the specific immune response to the etiologic agent. Boosting by repeated exposure to HSPs from a variety of microorganisms may assure constantly high levels of immunity. In other cases, however, the immune response to HSPs seems to be restricted to more specific epitopes [8]. Such unique epitopes, of course, would be potentially more useful for diagnostic purposes and represent more interesting candidates for vaccine development. This review will discuss the potential use of HSPs to vaccinate against mycobacterial infections. The first part of this work concerns expression of the mycobacterial HSP65 DNA from a retroviral vector that integrates into the cell nucleus of J774 macrophage-like tumor cells, making a transgenic cell line that produces the protein. We used this vector to vaccinate mice, and examined the immune response – learning a lot about it. We then learned that we could use naked DNA; thus, the second part of this paper concerns DNA vaccination using HSP DNA.

2. Signs of hope

Tuberculosis kills three million people every year [9, 10], due to respiratory infection with *M. tuberculosis*, and the World Health Organization places its hope in bringing tuberculosis under control on a combination of vaccination with bacillus Calmette-Guérin (BCG) to boost immunity and antibacterial drug treatment to directly kill the bacteria [9]. Despite these efforts, there are still 10 million new cases and 3 million deaths worldwide every year, mainly in developing countries, and this changes little from year to year [10]. The disease is now increasing again in the more affluent countries that have been so complacent about the situation elsewhere. Increasingly, the disease is caused by multidrug-resistant varieties of *M. tuberculosis*, and it is then not only highly infectious but also essentially incurable [10, 11]. HIV-infected people are exquisitely susceptible, and they represent an increasing fraction of the general population in all countries. Moreover, there are few signs of new antituberculosis drugs appearing in the near future, but there is real hope that recombinant DNA techniques will quickly produce major advances in the development of materials for vaccination [12].

The BCG vaccine is in widespread use against tuberculosis but it has doubtful impact on the global position. It is a live vaccine, derived from *Mycobacterium bovis* by virulence attenuation during prolonged cultivation of the bacteria in the laboratory. In ten randomized controlled trials of BCG vaccines carried out since 1930, the protective efficacy against tuberculosis has ranged from 0 to 80% in different populations [11, 13]. Thus, BCG is far from being an ideal vaccine against tuberculosis, and in several countries it is virtually unused, not only because of doubts about its efficacy but also because it prevents the subsequent use of skin sensitivity tests to detect tuberculosis infection [11]. Therefore, a new vaccine that contained only a few key protective antigens could be the answer.

The reductionist approach to the development of a new vaccine against tuberculosis has not given outstanding results. Over the years, it had been established that injection of dead BCG or a wide variety of antigenic components, even in large amounts and with adjuvant, generates only a moderate degree of protective immunity in experimental animals [14]. So the question had become one of defining the special feature(s) of live BCG, and there was reason to doubt whether any individual antigen could do what the live bacterium could do. However, the availability of cloned genes for mycobacterial protein antigens and expression vectors for mammalian cells has enabled a new approach – that of expressing the individual genes directly in antigen-presenting cells (APCs). The rationale for this approach is that BCG, like *M. tuberculosis*, multiplies inside macrophages, one of the main APCs of the immune system, and antigens that arise from within APCs can be presented for immune recognition differently and evoke different immune responses, from antigens coming from outside the cells [15]. Thus, endogenous antigens, from intracellular viruses or bacteria, can be presented on the cell surface for recognition by specialized cytotoxic T lymphocytes (CD8 T cells); exogenous antigen tends to be presented for recognition by helper T lymphocytes (CD4 T cells). Hence, we reasoned that if we took the genes for those antigens that are likely to be highly expressed by mycobacteria living in macrophages and expressed them in macrophages as if they were viral antigens, we might reproduce some essential features of the live BCG vaccine [16–21].

3. HSPs as antigens

A variety of antigens that are not prominent in early *M. tuberculosis* culture filtrate but may be released later are also frequently recognized by T cells from tuberculosis patients [22]. These include 19-kDa antigen [23], HSP10 [24], PhoS, HSP65 and HSP70 [12, 25]. The HSPs, in particular, have generated interest as potential protective antigens, largely on the basis of their prominence in the immune response in mice and humans at both antibody and T-cell level [2, 7, 24, 25]. Furthermore, HSP65 appears to be produced in increased amounts by intracellular tubercle bacilli [26], presumably in response to the stress of intracellular existence, and a 17-kDa antigen that is the homologue of the α-crystallin stress protein becomes a dominant mycobacterial protein under such conditions [27]. It is intriguing that the α-crystallin homologue and HSP65 and HSP10 have been identified as prominent immunogens in a mycobacterial ribosomal preparation [28] that represents one of the more successful of the earlier subunit vaccines [29]. Thus it seems likely that antigens such as stress proteins that are produced late in infection are also good targets for a protective immune response, particularly if the development of bacterial persistence and dormancy are to be overcome, and even more so, if a therapeutic vaccine is to be developed.

4. Immunization with a retroviral vector

We took the first cloned mycobacterial antigen gene to become available to us, that of the HSP65 of *M. leprae*. Fortunately, this protein is a highly conserved one, being antigenically very similar to that of *M. tuberculosis*, and seemed likely to be produced by mycobacteria under the stress of intracellular residence. Thus, the initial steps toward this realization were taken when we used a retroviral shuttle vector to express the HSP65 antigen in the monocyte-like tumor cell line J774 (Balb/c origin) [16]. The cells presented the expressed antigen for specific recognition by T cells of the $CD4^+$, $CD8^+$, and γδ T-cell receptor types [16–21]. When Balb/c mice were given a series of injections (i.p. or i.v.) of the transfected cells (J774-HSP65), they acquired a remarkably high degree of protection against a subsequent intraperitoneal or intravenous challenge infection with virulent *M. tuberculosis* H37Rv; bacterial numbers declined exponentially in internal organs and were, for example, 100-fold lower in lungs after five weeks than in control animals [18, 19]. Injection of the protein with adjuvant was ineffective. Protection was dependent on tumor cell viability and was antigen specific; for example there was no protection against

challenge with *Listeria monocytogenes* [17–19]. Although the immunizing J774-HSP65 cells rapidly disappeared in vivo, dead cells did not work and the protective effect was small in C57Bl/6 mice (haplotype b). This was consistent with protection depending on the function of the transfected cells as APCs, rather than as merely an antigen source [21].

It was really quite unexpected that we could obtain such a high degree of protection with this system, and it has caused a major conceptual shift: in effect, protection can be achieved by vaccinating with a single mycobacterial protein. In practice, we might be reluctant to base a vaccine on a single protein, and several may need to be included for the outbred human population, where individuals will not necessarily all respond to any one antigen. For this reason, we are testing genes for several other mycobacterial antigens. Cognizance must also be made of the presence in HSP65 of cross-reactive epitopes that are relevant to autoimmune diseases, and these might be better removed from a vaccine. The other main issue raised is whether similar protection can be achieved by hyperexpressing the gene in APCs other than tumor cells.

One way of avoiding the use of tumor cells is to remove normal tissue, introduce the gene into it in vitro, and replace the tissue. We have done this with bone marrow from 5-fluorouracil-treated BALB/c mice, using the same retroviral construct to transfect stem cells in culture and transplanting the cells into lethally γ-irradiated recipients [20]. A high proportion of the recipients were expressing HSP65 in their peripheral blood cells after two weeks, and about half of those had specific delayed-type hypersensitivity reactions to the protein (footpad swelling). Challenge infection by intravenous injection of *M. tuberculosis* H37Rv showed that those mice with delayed-type hypersensitivity reactions were protected, and those without were not. The degree of protection was similar to that which was obtained in the experiments using J774-HSP65: three weeks after infection, counts of viable bacteria in liver, spleen, and lungs were about two logs lower in delayed-type hypersensitivity responders than in non-responders or in mice reconstituted with normal bone marrow or with marrow transfected with the retroviral vector without the mycobacterial gene [20]. Evidently, features unique to tumor cells were not essential for the protective response, and we investigated whether the viruslike endogenous origin of the antigen synthesized by the transfected cell was the key.

5. The importance of a cytotoxic T-cell response

The salient feature of endogenous antigen is presentation on major histocompatibility complex (MHC) class I for recognition by T cells via CD8-associated receptors. As expected, limiting dilution analysis of T cells in the spleens of J774-HSP65-immunized mice showed that there was an increase in $CD8^+$ T cells that was at least equal to the $CD4^+$ response [18, 19, 21], and very high frequencies were reached (1:250). Protein in adjuvant, in contrast, selectively increased the frequency of $CD4^+$ cells. Furthermore, 12 $CD4^+$ and 12 $CD8^+$ HSP65-specific T-cell clones were characterized extensively and were shown to have antimycobacterial activity in vitro [21]. If supernatants from the clones were used to activate macrophages, they would activate to inhibit multiplication of intracellular tubercle bacilli and if the clones were put into cell-cell contact with infected macrophages in the presence of anti-IFN-γ, those cells that had antigen-specific cytotoxicity would inhibit the growth of the bacteria too. Thus, they had two properties that were associated with in vitro antimycobacterial activity: IFN-γ production and cytotoxicity. We used representative clones in adoptive transfer of protection [21]. These clones represented the spectrum of activities among $CD4^+$ and $CD8^+$ clones in terms of IFN-γ and IL-4 production and cytotoxicity. Some of the clones resulted in bacterial killing: there was a quite substantial reduction in numbers of bacteria in the internal organs. The most effective clones were $CD8^+$ cytotoxic clones. If the clone produced IFN-γ as well, we could neutralize the effect attributable to IFN-γ by giving the animal monoclonal antibody against IFN-γ. From this study, we concluded that $CD8^+$ clones probably are more effective than $CD4^+$ clones, and IFN-γ and cytotoxicity both contribute to protection, perhaps with cytotoxicity being more important [21].

Robert Modlin and coworkers [30] have recently revealed the existence, in man, of categories of cytotoxic $CD8^+$ T lymphocytes that deliver highly microbicidal proteins into infected macrophages. In so doing, they kill virulent *M. tuberculosis*. These lethal cells can also be conventionally MHC class I restricted. Their essential feature is that they lyse the target macrophages by the granule-mediated perforin mechanism of apoptosis and codeliver bactericidal proteins. A key mycobactericidal protein delivered by the T cells has been identified as granulysin, present in the same granules as perforin [31]. This protein is also found in NK cell granules and has known potent lethal action against a range of microorganisms and tumor cells.

6. Immunization with plasmid DNA

A disadvantage of the retroviral vector used to generate HSP65 inside APCs is that it is dependent on integration into the nucleus of multiplying cells, such as tumor or bone marrow stem cells. A much simpler and more versatile approach has now become available in the unlikely form of direct injection of plasmid DNA. This nucleic acid vaccination approach has dramatically facilitated the global quest for new vaccines [32], and we have found that it is highly effective against tuberculosis [33–37]. Thus, the DNA vaccination is an alternative means of generating endogenous antigen, and at the first WHO DNA vaccine meeting in 1994, we reported the first evidence that this too could be used to generate protective antituberculosis immunity [33].

For nucleic acid vaccination, we have used plasmids in which the HSP65 DNA is expressed either from the cytomegalovirus immediate-early gene promoter (pCMV) or from the murine hydroxymethylglutaryl-CoA reductase

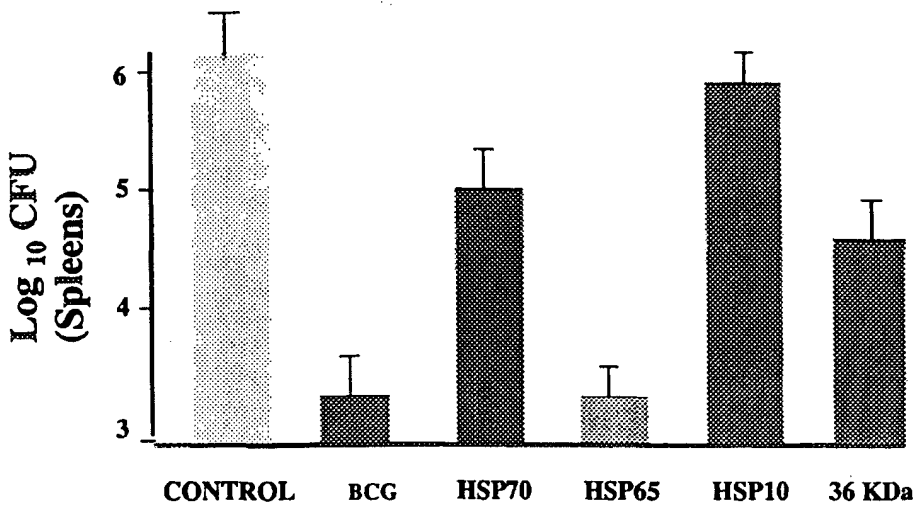

Figure 1. Comparison of protection against tuberculosis by vaccination with DNA encoding individual mycobacterial antigens.

gene (pHMG), with similar results [33]. Most importantly, we have confirmed that immunization with DNA encoding only a single mycobacterial antigen can confer protection equal to that obtained with live BCG. In a typical test, we immunize by injecting mice with 50 μg of DNA expressing HSP65 into the muscle of each hind leg four times at 2- to 3-week intervals; we then challenge by intraperitoneal infection with virulent *M. tuberculosis* three weeks later and assess the progress of the infection five weeks after that by counting the live bacteria in internal organs. Compared with nonimmunized control animals or those that receive plasmid DNA that does not contain the mycobacterial gene, mice immunized with HSP65 DNA have about 100-fold fewer bacteria in the lungs. This is very similar to the protective effect obtained in mice given intradermal BCG and tested in parallel [33–37].

HSP65 DNA protects to a similar extent all the mouse strains we have tested so far: BALB/c, outbred albino Parkes and cross-bred CBA/B10. Broad protection is obviously advantageous in a candidate for use in a practical vaccine and presumably reflects the multiple and promiscuous epitopes on the antigen [33–37]. However, a practical vaccine would probably contain more than one antigen, or as we now think, the genes for more than one antigen, and we are using this approach to screen other candidates for inclusion. We have now tested five known mycobacterial protein antigens individually or in combination as DNA vaccines using pCMV constructs in BALB/c mice. Effective genes were those encoding HSP65, HSP70, or 36-kDa proline-rich antigen, and the degree of protection could equal that obtained with live BCG [33-37] *(figure 1)*. When we mix plasmids together and inject them simultaneously we can get greater protection than when they are injected individually [34].

Antibody responses were readily detected by ELISA two weeks after the third dose of plasmid expressing HSP65, and strong lymphoproliferative responses to HSP65 antigen were also found in splenocytes [34–36]. Cells responding after 4-day culture with the antigen released IFN-γ but no detectable IL-4 (ELISAs), indicating a predominantly Th1/Tc1-type response. IL-4 (an indicator of a Th2-type response) became detectable in vitro after vaccinating with plasmid encoding accessory molecule B7.2 in a 50:50 mixture with HSP65 DNA [34]. The strong Th1/Tc1 bias of the response was further evidenced by reverse transcription-PCR analysis of mRNA for cytokines in inguinal lymph nodes draining HSP65-DNA-vaccinated muscle two weeks after the fourth injection; no mRNA for IL-4, IL-10, or IL-13 was detectable, whereas IFN-γ and IL-12 mRNA were increased relative to controls receiving empty plasmid DNA. Splenocytes from HSP65 DNA-vaccinated mice also displayed antigen-specific cytotoxicity against ^{51}Cr-loaded P815 target cells that had been pulsed with synthetic peptide. Two out of three peptides representing predicted MHC class I restricted T-cell epitopes of HSP65 were recognized [34].

7. Persistence of memory for specific cytotoxicity after DNA vaccination

In an experiment comparing different means of immunizing with HSP65, we have now established that protocols that generate the antigen endogenously (J774-HSP65 or DNA vaccination) or that deliver the antigen into the cytosol (protein-loaded liposomes) generate protection and this is associated with a strong $CD8^+$ response in which $CD44^{hi}$ memory-associated cells are prominent [37]. The greatest protection was seen one week after immunization with J774-HSP65, but this declined substantialy by eight months. Liposomes and DNA vaccination also gave substantial early protection equal to that of BCG, and whereas protection after liposomes declined, as did protection after J774-HSP65, protection after HSP65 DNA was sustained, as in BCG-induced protection. DNA vaccination also induced a sustained increase in the proportion of $CD8^+$ splenocytes that were $CD44^{hi}$. The frequencies of cytotoxic HSP65-responsive cells among

$CD44^{hi}/CD8^{+}$ splenocytes at different intervals after the various vaccination protocols show that at one week postimmunization, the highest frequency was seen with J774-HSP65, reaching one in eight, and this had substantially declined by eight months postimmunization. HSP65-liposomes and HSP65 DNA also resulted in a high frequency of $CD44^{hi}$ cells at one week (about one in 200), and this subsequently declined in the case of HSP65-liposomes but increased to one in 12 by eight months, in the case of DNA vaccination. This rising frequency of cytotoxic HSP65-specific $CD8^{+}$ memory cells was also seen after BCG vaccination. One hypothesis to account for these findings is that only live BCG and DNA vaccination provide a persistent source of intracellular antigen and this is needed to sustain the cytotoxic T-cell memory that is required for long-lasting protection.

8. Type-1 cytokines

A striking difference between the immune response to DNA vaccination and the immune response to either BCG or *M. tuberculosis* infection is that DNA induces almost entirely a protective type-1 cytokine response, whereas the mycobacterial infections have a major component of noncytotoxic T cells that produce a noncharacteristic type-1 cytokine response [37]. During infection or after immunization, HSP65-reactive CD4 and CD8 T cells increased equally in spleens. During infection, the majority of these cells were $CD44^{lo}$ and produced IL-4, whereas after immunization, the majority were $CD44^{hi}$ and produced IFN-γ. In adoptive transfer of protection to naive mice, the total CD8 cell population purified from spleens of immunized mice was more protective than that from infected mice. When the cells were separated into CD4 and CD8 types and then into $CD44^{hi}$ and $CD44^{lo}$ types, $CD44^{lo}$ cells were essentially unable to transfer protection, the most protective $CD44^{hi}$ cells were CD8, and those from immunized mice were much more protective than those from infected mice. Thus, whereas the $CD44^{lo}$ IL-4-producing phenotype prevailed during infection, protection was associated with the $CD8/CD44^{hi}$ IFN-γ-producing phenotype that predominated after immunization. This conclusion was confirmed and extended by analysis of 16 HSP65-reactive T-cell clones from infected mice and 16 from immunized mice; the most protective clones, in addition, displayed antigen-specific cytotoxicity [37].

9. HSPs as adjuvants

Many of the recombinant or synthetic antigens now being considered as vaccine candidate antigens are not sufficiently immunogenic by themselves to induce a strong and protective immune response. In order to improve the immunogenicity of these preparations, the addition of an immunostimulant (i.e., adjuvant) is required. Unfortunately, a major obstacle for the development of effective new vaccines is the lack of optimal adjuvants acceptable for human use. Aluminium oxide is the most widely used adjuvant in humans, but it is limited with respect to the type of antigen employed and to the stability of the final vaccine preparation. Hence, many different substances and strategies destined to enhance the human response to immunogens are being explored as alternative adjuvants. Several studies [38] have demonstrated that covalent complexes of mycobacterial HSP65 or HSP70 and peptides could be used to elicit potent and specific anti-peptide antibodies without the use of additional adjuvants. The responses were shown to be T-cell dependent. Cytotoxic T lymphocyte responses and T-cell-dependent protective immunity have been elicited recently by immunization of mice with a mycobacterial HSP70-ovalbumin fusion protein. Clearly, these and other studies show that immunization with HSP-peptide complexes can elicit not only cytotoxic T lymphocytes but also antibody responses.

The adjuvanticity of mammalian HSPs is unique in many respects. They are the first, and thus far, the only, adjuvants of mammalian origin. Being self-antigens, they do not elicit an immune response to themselves. They are nonlive and yet they elicit CD8 T-cell responses in addition to antibody responses, and finally, their apparent promiscuity in peptide-binding makes them applicable to a wide variety of antigens. These properties are particularly interesting in light of the paucity of adjuvants judged to be effective and safe for human use.

10. Concluding remarks

These results indicate that the very high level of protective efficacy of J774-HSP65 or HSP65-DNA vaccination against tuberculosis can be ascribed to two features of the immune response: induction of HSP65-reactive T cells to a very high frequency in the lymphocyte population and induction of cells with an appropriate phenotype. These findings have implications both for understanding the mechanisms of protection and developing practical vaccines.

It can be argued that microbial stress proteins do not make good candidates for inclusion in subunit vaccines because of the implication of the homologous mammalian proteins in autoimmune diseases [2]. However, there are possible alternatives to leaving these major antigens out of vaccines: the epitopes involved might be readly engineered out of the DNA, or the formulation and delivery might be selected to direct the response away from a harmful type and towards a beneficial one. Indeed, T cells recognizing mycobacterial HSPs can either protect against or potentiate autoimmune disease, depending on their phenotype [39, 40]. Our group recently showed that DNA vaccination with mycobacterial HSP65 protected Lewis rats against induction of adjuvant arthritis [41]. This may be the best approach in the long run because, to the extent that an acquired immune response depends on recognition of 'altered self' rather than 'foreign', essentially all antigens may have the potential for autoimmune involvement.

Others have argued on theoretical grounds that the immune recognition of HSP65 is irrelevant to protective immunity [42]. The argument seems to be i) that the

antigen will not be released by live bacteria in infected cells but will only appear late in the infection when the bacteria are being killed following immune recognition of some other antigen(s), and then ii) that the phenotype of the T cells generated at that stage is not appropriate for protection. Our experiments show that both this conclusion and it premises are mistaken. Regarding antigen availability, we see that the prominence of $CD8^+$ HSP65-reactive T cells in BCG-vaccinated mice, the lysis of infected macrophages by such T cells in vitro, and the protective effect in vivo on adoptive transfer all imply that the HSP65 protein is produced in substantial amounts by intracellular mycobacteria. It is possible that cross-reactive endogenous murine HSP60 induced by the stress of infection could also play a part [43]. However, we previously showed by competitive inhibition that the cytotoxic activity of $CD8^+$ clones was specific for mycobacterial HSP65 [18, 19]. In conclusion, the immunogenicity of HSPs and HSP-peptide complexes has a number of significant implications for vaccination against intracellular infections and treatment of preexisting infections caused by viruses, mycobacteria, and certain parasites.

Acknowledgments

The author thanks Vania L.D. Bonato, Valéria M.F. Lima, and Douglas B. Lowrie for their fruitful collaboration, and Izaira Tincani Brandão for technical assistance. Studies carried out in Brazil reported in this article were supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Financiadora de Estudos e Projetos (FINEP).

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7218980

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Stress-Inducible Cellular Responses
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Heat shock proteins as immunological carriers and vaccines

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Summary. HSPs are among the major targets of the immune response to bacterial, fungal and parasitic pathogens. The antigenic nature of HSPs is emphasized by evidence that mammals are capable of recognizing multiple B- and T cell epitopes in these proteins. The powerful immunological features of HSPs have led to their experimental use as immunomodulators and as subunit vaccine candidates. Mycobacterial hsp70 and hsp60 have been found to be excellent immunological carriers of molecules against which an immune response is desired; in the absence of adjuvants, the HSPs can stimulate strong and long-lasting immune responses against molecules which have been covalently attached to the HSPs. When used as subunit vaccines, HSPs derived from a variety of bacterial and fungal pathogens have been found to stimulate protective immunity in animal models. These studies suggest that HSPs might be used as immunomodulators or subunit vaccines against infectious disease in man.

Introduction

Humans are exposed to a large number and variety of pathogenic and non-pathogenic microorganisms. Our bodies provide niches for a multitude of microbes that do not typically cause disease. For example, approximately 10^6 cocci and diptheroids reside on one cubic centimeter of human skin (Leyden et al., 1991) and 1 ml of saliva contains about 10^8 microbes (Rosebury, 1962). We are also continuously confronted by pathogenic organisms of substantial diversity.

To protect us from these microorganisms, the immune system has evolved to recognize a remarkable variety of antigenic determinants. Despite this capacity, the immune system appears to devote considerable attention to the members of one family of proteins, the ubiquitous heat shock proteins (HSPs) (Young, 1990; Kaufmann, 1990; Young et al., 1990; Murray and Young, 1992; Kaufmann and Schoel, 1994). The HSPs are among the major targets of the immune response to most bacterial and parasitic pathogens. Humoral and cellular immune responses to HSPs have been observed following exposure to a broad spectrum of infectious agents, including gram positive and gram negative bacteria, fungi, helminths and protozoa (Tab. 1).

The immune responses to HSPs elicited by mycobacterial pathogens have been particularly well-studied. Exposure to *Mycobacterium tuber-*

Table 1. Pathogens induce immune responses to HSPs

Infectious agent	Disease	HSP	References
Bacteria			
Bordetella pertussis	pertussis	hsp70, hsp60	Del Giudice et al., 1993
Borrelia burgdorferi	Lyme disease	hsp60	Hansen et al., 1988
Brucella abortus	brucellosis	hsp60	Roop, et al., 1992
Chlamydia trachomatis	blinding trachoma	hsp70, hsp60	Taylor et al., 1990; Cerrone et al., 1991; Zhong and Brunham, 1992
Coxiella burnetti	Q fever	hsp60	Vodkin and Williams, 1988
Helicobacter pylori	gastritis	hsp60, hsp10	Suerbaum, 1994, Ferrero et al., 1995
Legionella pneumophila	Legionnaires' disease	hsp60	Hoffman et al., 1990
Mycobacterium leprae	leprosy	hsp70, hsp60, small hsps	Mehra et al., 1992; Young et al., 1988
Mycobacterium tuberculosis	tuberculosis	hsp70, hsp60, small hsps	Young et al., 1988, Shinnick et al., 1988; Baird et al., 1988
Treponema pallidum	syphilis	hsp60	Hindersson et al., 1987
Fungi			
Aspergillus fumigatus	aspergillosis	hsp60	Kumar et al., 1993
Candida albicans	candidasis	hsp90	Matthews et al., 1987; Matthews and Burnie 1989
Histoplasma capsulatum	histoplasmosis	hsp60, hsp70	Gomez et al, 1991a; 1992, 1995
Helminths			
Brugia malayi	lymphatic filariasis	hsp70	Selkirk et al., 1989
Onchocerca volvus	ocular filariasis	hsp70	Rothstein et al., 1989
Schistosoma mansoni	schistosomiasis	hsp90, hsp70, small hsps	Johnson et al., 1989; Hedstrom et al., 1987; Nene et al., 1986
Schistosoma japonicum	schistosomiasis	hsp70	Scallon et al., 1987; Hedstrom et al., 1988
Protozoa			
Leishmania braziliensis	leishmaniasis	hsp70	Levy Yeyati et al., 1992
Leishmania donovani	visceral leishmaniasis	hsp90, hsp70	MacFarlane et al., 1990; de Andrade et al., 1992
Plasmodium falciparum	malaria	hsp70	Mattei et al., 1989; Renia et al., 1990
Trypanosoma cruzi	Chagas' disease	hsp70	Levy Yeyati et al., 1992; Requena et al., 1993

culosis or *Mycobacterium leprae* leads to humoral and cellular immune responses to hsp70, hsp60 and small hsps (18kD, 14kD, 10kD) (Adams et al., 1990; Husson and Young, 1987; Young et al., 1988; Nerland et al., 1988; Verbon et al., 1992; Mehra et al., 1992). The cellular responses to mycobacterial HSPs are profound; limiting dilution analysis indicates that 20% of the murine $CD4^+$ T lymphocytes that recognize mycobacterial antigens are directed against hsp60 alone (Kaufmann et al., 1987). The high frequency with which human $CD4^+$ T cells directed against mycobacterial hsp70 and hsp60 have been detected suggest that these HSPs are also major targets of the cellular response in humans (Munk et al., 1988). Limiting dilution analysis of human T lymphocytes from a tuberculoid leprosy patient as well as a patient contact revealed that one-third of *M. leprae* reactive T cells were directed against hsp10 (Mehra et al., 1992).

The powerful antigenic nature of HSPs is emphasized by evidence that mammals are capable of recognizing multiple B and T cell epitopes in these proteins. Murine and human B cell epitopes have been mapped in HSPs from *M. tuberculosis, M. leprae, Trypanosoma cruzi* and *Plasmodium falciparum* (Mehra et al., 1986; Thole et al., 1988; Richman et al., 1989; Mattei et al., 1989; Requena et al., 1993). These mapping data indicate that B cells can recognize many portions of the hsp70 and hsp60 protein molecules. Murine and human T cell epitopes have been mapped most extensively for mycobacterial hsp60 and hsp70 (Lamb et al., 1987; Munk et al., 1990; Van Schooten et al., 1988; Adams et al., 1994; Oftung et al., 1994). This evidence indicates that mycobacterial HSPs can be presented in the context of multiple MHC haplotypes, and that T cell epitopes can be found throughout these HSPs.

The powerful immunological features of HSPs have led to their experimental use as immunomodulators and as subunit vaccine candidates. Mycobacterial hsp70 and hsp60 have been found to be excellent immunological carriers of molecules against which an immune response is desired; in the absence of adjuvants, the HSPs can stimulate strong and long-lasting immune responses against molecules which have been covalently attached to the HSPs. When used as subunit vaccines, HSPs derived from a variety of pathogens have been found to stimulate protective immunity in animal models. We describe below the concepts behind immunological carrier proteins and adjuvants, and then discuss the adjuvant-free carrier effects of hsp60 and hsp70. We then review evidence supporting the use of HSPs as vaccines against specific pathogens.

Immunological carrier proteins and adjuvants

Many polysaccharides and simple chemical compounds are inherently non-immunogenic and fail to elicit strong antibody responses. Landsteiner (1936) observed that these substances, "haptens", react *in vitro* with anti-

bodies but do not have the capacity to elicit antibodies *in vivo*. However, if the hapten was administered in combination with a "carrier" protein, antibodies to the hapten could be generated.

Ovary and Benacerraf (1963) demonstrated that the same carrier protein used in the primary immunization must be used in the subsequent immunization in order to elicit a secondary immune response to the hapten. The hapten and carrier had to be physically linked and within this conjugate molecule, cells recognized one antigenic determinant on the hapten and a second determinant on the carrier (Mitchison, 1971a; Rajewsky et al., 1969). It was determined that in the generation of an antibody response, two distinct types of cells were involved: bone marrow derived lymphocytes (B cells) and thymus derived lymphocytes (T cells) (Claman et al., 1966; Davies et al., 1967; Miller and Mitchell, 1967). Involvement of B and T cell cooperation in the "carrier effect" was demonstrated by adoptive transfer experiments in which one mouse was injected with the hapten, a second mouse was injected with the carrier, and a third mouse was irradiated and received hapten-primed B cells and carrier-primed T cells (Mitchison, 1971b; Raff, 1970). The B and T cells could collaborate to generate an antibody response only if they were obtained from syngeneic mice (Kindred and Shreffler, 1972; Katz et al., 1973).

The "carrier effect" is believed to occur in the following manner. When animals are primed with a hapten-carrier preparation, and then exposed to a second dose, hapten-specific B cells recognize, internalize, and process the hapten-carrier conjugate. The B cell can then present peptides derived from the carrier molecule on its surface in the context of an MHC molecule. This MHC/peptide complex is bound by a carrier-primed T cell, leading to the direct release of cytokines by the T cell to the B cell. These soluble factors stimulate the B cell to proliferate, differentiate, and secrete antibodies. In this review, a "carrier" will refer to a molecule containing T cell epitopes which, when covalently linked to a second molecule, help to elicit and enhance immune responses against the second molecule.

Carriers are an important component of some human vaccines. Tetanus toxoid (TT), diphtheria toxoid (DT) and neisseria outer membrane proteins are the carrier molecules used in the various *Haemophilus influenza* vaccines licensed for use in humans (Smith et al., 1989). In these vaccines, the principal virulence determinant of *H. influenza* type b, a repeating polymer of ribose, ribitol and phosphate, had to be conjugated to a carrier in order to elicit the high levels of anti-polysaccharide antibodies necessary for protective immunity in young children (Robbins and Schneerson, 1990).

The immune response to an antigen of interest can also be enhanced by use of an adjuvant. Indeed, adjuvants are often necessary to elicit desired immune responses (Geerligs 1989; Kenney et al., 1989). In contrast to a carrier, an adjuvant does not need to be covalently coupled to the antigen to perform its function. Instead, the adjuvant and antigen are absorbed or

mixed together and are co-administered (Nicklas, 1992). In general, adjuvants function by slowly releasing the antigen, thereby acting as a long-lived antigen reservoir (called the depot effect), and by causing general inflammation at the injection site and thus recruiting immunological mediators such as macrophages. Many adjuvants containing bacterial components, oils and various chemicals have been described (Edelman and Tacket, 1990).

Alum, which contains the aluminum salts $Al(OH)_3$ and $AlPO_4$, is currently the only adjuvant licensed for use in humans and is included in vaccines against diphtheria, tetanus, pertussis, *H. influenza* and hepatitis B. However, alum is poor at stimulating cell-mediated responses and is not effective in the induction of humoral responses against certain antigens (Altman and Dixon, 1989). Thus, a considerable effort is being made to develop new safe and effective adjuvants for use in man (Lussow et al., 1990a; Dintzis, 1992; Audibert and Lise, 1993).

Adjuvant-free carrier effect of hsp60 and hsp70 proteins

Purified protein derivative (PPD), prepared from mycobacterial culture supernatant, is a protein mix which contains hsp60 and hsp70 and elicits a delayed type hypersensitivity reaction in individuals previously exposed to mycobacteria. The powerful immunostimulatory properties of PPD also suggested that it might have some utility as an immunological carrier. Indeed, when PPD was cross-linked to small chemical haptens or peptides, the conjugates elicited a strong antibody response against the attached molecules (Lachmann and Amos, 1970; Lachmann et al., 1986). In addition, a PPD-tumor cell conjugate has been found to enhance the immune response to tumor cells (Lachmann and Sikora, 1978). For PPD to be an effective carrier, physical linkage of PPD to the antigen was crucial and carrier priming with bacille Calmette-Guerin (BCG) was necessary. The powerful carrier effect of PPD was also evident in comparative studies using various conjugates administered with Freund's adjuvant; these studies demonstrated that PPD was a more effective carrier than bovine serum albumin or keyhole limpet hemacyanin (Lachman et al., 1986). PPD has been shown to be an effective carrier in the absence of adjuvant. When the synthetic malarial peptide $(NANP)_{40}$, an epitope from the *Plasmodium falciparum* major surface protein, was conjugated to PPD and administered in Freund's adjuvant or in saline to BCG primed mice, the anti-$(NANP)_{40}$ antibody titers were equivalent (Lussow et al., 1990b).

Recombinant, mycobacterial hsp60 and hsp70 proteins can substitute for PPD in a $(NANP)_{40}$ conjugate (Lussow et al., 1991), suggesting that these were among the components of PPD responsible for the adjuvant-free carrier effect. Hsp60-$(NANP)_{40}$ and hsp70-$(NANP)_{40}$ conjugates were found to elicit anti-$(NANP)_{40}$ antibodies in mice and squirrel monkeys in

the absence of adjuvants (Lussow et al., 1991; Barrios et al., 1992; Perraut et al., 1993). Moreover, mycobacterial hsp60 and hsp70 were also found to be effective adjuvant-free carriers when conjugated to the poorly immunogenic meningococcal group C oligosaccharide (MenC) (Barrios et al., 1992).

Other results from the $(NANP)_{40}$ studies support the notion that HSPs can be powerful carriers, but indicate that not all HSPs behave identically. $(NANP)_{40}$ alone acts as a hapten in most strains of mice (Good et al., 1986; Del Giudice et al., 1986), and the presence of covalently linked mycobacterial hsp60 or hsp70 carrier molecules was essential to obtain antibody responses against $(NANP)_{40}$ (Lussow et al., 1990b). The corresponding heat shock proteins from *E. coli*, GroEL and DnaK, could also function as adjuvant-free carriers to elicit anti-$(NANP)_{40}$ antibodies (Barrios et al., 1994). However the hsp70 conjugates exhibited one useful feature that was not observed with hsp60 conjugates. While priming with recombinant hsp60 in Freund's adjuvant or with living BCG was necessary to obtain the carrier effect with the hsp60 conjugates, but priming was unnecessary with the hsp70 conjugates. Moreover, previous exposure to BCG or to hsp70 neither augmented nor suppressed the antibody response against the antigen attached to hsp70.

To further investigate the adjuvant-free carrier effect of mycobacterial hsp70, an hsp70 fusion vector system was created (Suzue and Young, 1996). This system enabled the production of proteins composed of one mole of antigen fused to one mole of the hsp70 carrier protein in which the number and position of potential epitopes were identical for each molecule. In contrast, hsp70 conjugates generated by glutaraldehyde cross-linking are a pool of nonidentical molecules with variable epitope density. Immune responses to an antigen can be strongly affected by differences in the molar ratio of antigen and carrier, in the mode of linkage of hapten and carrier and in the position of B and T cell epitobes (Hanna et al., 1972; Klaus and Cross, 1974; Snippe et al., 1975; Anderson et al., 1989; Dintzis, 1992). Thus, hsp70 fusion proteins reduce the variables associated with the study of HSPs as immunological carriers.

The hsp70 fusion vector system was used to produce an HIV Gag-hsp70 fusion protein and to investigate the humoral and cellular immune response to it (Suzue and Young, 1996). The mycobacterial hsp70 moiety was found to dramatically increase the immunogenicity of the Gag p24 antigen. Mice immunized with the p24-hsp70 fusion protein in phosphate buffer generated a vigorous humoral immune response against p24, whereas administration of p24 elicited low levels of anti-p24 antibody wich was not long lasting. The high level of anti-p24 antibody elicited by the p24-hsp70 fusion protein was long-lived; high titres of anti-p24 antibodies were detected up to 68 weeks after immunization. The p24-hsp70 fusion protein also stimulated T cell responses to p24 in the absence of adjuvant. Splenocytes from mice immunized with the fusion protein exhibited p24 anti-

gen-dependent proliferation and production of the cytokines IFN-γ, IL5 and IL-2. Covalent linkage of hsp70 to p24 was essential in order for hsp70 to exert an adjuvant-free carrier effect with p24. Thus, under these conditions, hsp70 is not a conventional adjuvant, in that its administration together with an unlinked antigen does not stimulate immune responses to that antigen. Instead, hsp70 functions as an exceptional carrier, since it stimulates vigorous immune responses when covalently linked to the antigen of interest. The reason that hsp70 is an exceptional carrier, and the reason that the hsp70 carrier does not require priming, is perhaps due to previous and ongoing exposure to substantial amounts of diverse microbial HSPs. It is also possible that the protein chaperone function of hsp70 contributes to the adjuvant-free carrier effect.

The above studies demonstrate that HSPs can act as adjuvant-free carriers when attached to a synthetic peptide, oligosaccharide or full-length protein. HSP fusion proteins and conjugates can induce strong humoral and cellular immune responses to a linked antigen. Thus, HSPs appear to be an important addition to the small repertoire of carriers available for the development of new vaccines.

HSPs as vaccines

HSPs from bacterial and fungal pathogens are among the major targets of the immune response to infection and have been found to be capable of inducing protective immunity in experimental animal models (Tab. 2). We

Table 2. HSPs can elicit protective immune responses

Infectious agent	HSP	Animal model	References
Bacteria			
Helicobacter pylori	hsp60, hsp10	mouse	Ferrero et al., 1995
Legionella pneumophila	hsp60	guinea pig	Blander and Horwitz, 1993
Mycobacterium leprae	hsp60, hsp10	mouse	Gelber et al., 1992; 1994
Mycobacterium tuberculosis	hsp70	mouse, guinea pig	Pal and Horwitz, 1992; Hubbard et al., 1992; Andersen 1994; Horwitz et al., 1995
Mycobacterium tuberculosis	hsp60	mouse	Silva et al., 1994a, 1994b; Lowrie et al., 1994
Yersinia enterocolitica	hsp60	mouse	Noll et al., 1994
Fungi			
Candida albicans	hsp90	mouse	Matthews et al., 1991
Histoplasma capsulatum	hsp60, hsp70	mouse	Gomez et al., 1991a, 1991b; 1992, 1995

review below examples of pathogenic microorganisms for which there is evidence that the immune response to HSPs contributes to protection against infection and disease.

The ability of *Legionella pneumophila* hsp60 to protect against Legionnaires' disease has been examined in a guinea pig model (Blander and Horwitz, 1993). Immunization of guinea pigs with purified *L. pneumophila* hsp60 was found to protect the animals from a lethal aerosol challenge with the organism. Cell-mediated immunity is known to be critical to the host defense against intracellular *L. pneumophila* (Horwitz, 1983) and animals immunized with purified *L. pneumophila* hsp60 exhibited delayed-tpye hypersensitivity (DTH) to hsp60, indicating that cellular responses had been elicited by the experimental vaccine.

M. tuberculosis hsp70 can contribute to protective immunity in animal models of infection. Immunizing mice and guinea pigs with *M. tuberculosis* culture filtrate proteins prior to challenge with the bacterium significantly reduced the number of organisms in the lung and spleen (Pal and Horwitz, 1992; Hubbard et al., 1992; Andersen, 1994). The colony forming units of mycobacteria recovered from the spleen after challenge was reduced by more than 95%, and even 22 weeks after vaccination, T cells from immunized mice could transfer protection to recipient mice (Andersen, 1994). Subsequently, abundant proteins in the *M. tuberculosis* culture filtrate were purified and analyzed for immunoprotective activity (Horwitz et al., 1995). Immunization of guinea pigs with purified hsp70 alone induced protection in some instances while a combination of five purified mycobacterial proteins, which included hsp70, consistently protected guinea pigs against weight loss and lung destruction when the animals were challenged with *M. tuberculosis*.

In contrast to hsp70, *M. tuberculosis* hsp60 does not elicit protective responses when administered as a soluble protein. However, vaccination of mice with syngenic J774 macrophage cells expressing mycobacterial hsp60 afforded remarkable protection against *M. tuberculosis* (Silva and Lowrie 1994). In mice vaccinated with J774-hsp60, 100 times fewer *M. tuberculosis* CFUs could be recovered from the liver 5 weeks after challenge, compared to unvaccinated mice. Hsp60 specific T cells cloned from the vaccinated mice could adoptively transfer protection to non-vaccinated mice (Silva et al., 1994). Vaccination of mice with mycobacterial hsp60 was also effective when administered to mice as a naked DNA vaccine (Lowrie et al., 1994). These studies indicate that eliciting protective immune responses can depend on the method of administration of an antigen. The *ex vivo* cell vaccine and the naked DNA vaccine approaches may better mimic an aspect of normal macrophage infection by *M. tuberculosis* than inoculation with a soluble antigen.

HSPs are also among the antigens of *Mycobacterium leprae* that appear to contribute to immunological protection. In order to identify protective components of *M. leprae*, mice were injected with various fractions deriv-

ed from the organism (Gelber et al., 1992). Vaccination of mice with a soluble protein fraction afforded significant protection against *M. leprae* infection and was more effective than administering killed *M. leprae* organisms. Studies were then carried out with recombinant *M. leprae* hsp60 and hsp10 to determine whether the highly protective nature of the soluble protein fraction could be attributed to a particular protein (Gelber et al., 1994). By challenging mice with *M. leprae* at different time intervals following vaccination, the extent of protection provided by each protein was determined. The soluble protein fraction provided protection and inhibited *M. leprae* growth up to one year after vaccination; hsp10 provided protection up to 2 months later and hsp60, up to 4 months. These experiments suggest that a cocktail containing multiple *M. leprae* proteins may be necessary to confer long-lasting protective immunity.

The inclusion of *Helicobacter pylori* HSPs in experimental vaccine preparations has been found to contribute to protection against gastroduodenal disease in a mouse model of *H. pylori* infection (Ferrero et al., 1995). Oral administration of recombinant *H. pylori* hsp60 or hsp10 protected a significant percentage of mice from infection. Immunization with both hsp10 and UreB, a urease subunit protein, conferred complete protection to the mice, and was found to be as effective as administering sonicated heliobacterial whole-cell extract.

HSPs have also been implicated in immunological protection against major fungal diseases. Immunization with purified *Histoplasma capsulatum* HSPs has been found to induce protective immunity against the pathogenic fungus in a mouse model of infection. Early experiments revealed that a detergent cell wall/cell membrane (CW/M) extract of *H. capsulatum* injected into mice could confer protection against a lethal challenge of yeast cells (Gomez et al., 1991 a). Further analysis revealed that a 62 kD protein component of the CW/M extract (HIS-62) was a target of *H. capsulatum*-specific T cells and was sufficient to engender protection against a lethal challenge (Gomez et al., 1991 b). Cloning of HIS-62 led to its identification as *H. capsulatum* hsp60 (Gomez et al., 1995). In contrast to the fortuitous identification of hsp60 as a protective antigen, hsp70 from *H. capsulatum* was identified and isolated with the intent of studying its involvement in immunity. Purified *H. capsulatum* hsp70 could induce DTH responses and protect mice from a sublethal injection of yeast cells (Gomez et al., 1992). Immunization with hsp60 confers protection against a higher challenge dose of *H. capsulatum* than does immunization with hsp70. It will be interesting to determine whether a vaccine consisting of both hsp60 and hsp70 might afford even better protection against *H. capsulatum*.

The significance of anti-HSP immune responses in some bacterial and fungal diseases has been affirmed in passive immunization studies. For example, the bacterial pathogen *Yersinia enterocolitica* elicits humoral and cellular responses against hsp60 in mice (Noll et al., 1994). The relevance

of HSP specific T cells in protective immunity against murine yesiniosis was examined by intravenously transferring 10^7 hsp60 specific T cell clones into naive mice. Three different T cell clones were examined and all of the passively immunized mice were protected when challenged with a lethal dose of pathogen. In studies with *Candida albicans*, hsp90 has been identified as an immunodominant antigen, and passive administration of an hsp90 monoclonal antibody was found to confer protection against systemic candidosis (Matthews et al., 1991).

The immune responses against HSPs confer protection against a broad range of pathogens. Although there is some concern with using HSPs in vaccine formulations due to their highly conserved nature and homology with self-HSPs, it must be emphasized that healthy individuals are routinely stimulated to respond to HSPs without causing autoimmunity. For example, the trivalent vaccine against tetanus, diphtheria and pertussis, which is routinely administered to infants, induces anti-hsp70 immune responses (Del Giudice et al., 1993). Live BCG, which contains substantial amounts of hsp70 and hsp60, has been used to immunize 80% of the world's children against tuberculosis. Thus, current knowledge suggests to us that the inclusion of HSPs in vaccines against a broad spectrum of infectious diseases would be both safe and beneficial.

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seminars in **CELL & DEVELOPMENTAL BIOLOGY**, Vol 10, 1999: pp. 495–505
Article No. scdb.1999.0320, available online at http://www.idealibrary.com on IDEAL®

GRP94, an ER chaperone with protein and peptide binding properties

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GRP94 is the ER representative of the HSP90 family of stress-induced proteins. It binds to a limited number of proteins in the secretory pathway, apparently by recognizing advanced folding intermediates or incompletely assembled proteins. GRP94 also binds peptides and can act as a tumor vaccine, delivering the peptides for presentation to T lymphocytes. Here, we review the current data about GRP94 and propose a structural model that integrates the biochemical data and known functions of the protein.

Key words: antigen presentation / HSP90 / peptide binding / protein folding / secretory pathway

Introduction

GRP94 IS THE ENDOPLASMIC RETICULUM (ER) resident member of the HSP90 family. It is among the most abundant of ER proteins, accounting for 5–10% of the lumenal contents with an estimated concentration of 10 mg/ml.[1] Unlike other abundant lumenal proteins, such as BiP, PDI or calreticulin, the physiological roles of GRP94 are enigmatic and its modes of action far less understood. This is even reflected in the somewhat confusing array of names for this protein. It was first described as one of a small set of proteins induced by glucose starvation of Rous Sarcoma Virus-transformed chick embryo fibroblasts,[2] hence the acronym GRP (glucose regulated protein). It was independently isolated as the most abundant calcium binding protein in the lumen and named endoplasmin[1] or CaBP4.[3] This protein was also recognized as one of three most induced in the ER of differentiating B lymphocytes, and termed ERp99.[4] In chicken, HSP108 was discovered as a heat shock protein involved in steroid induction of the oviduct and then shown to be identical to GRP94.[5,6] Finally, research into tumor rejection identified it as gp96, a glycoprotein which was a major tumor rejection antigen.[7]

GRP94 has recently attracted interest on two disparate fronts: its potential role as a molecular chaperone during folding of secretory and membrane proteins, and its activity and utility as a peptide carrier for T cell immunization. As described below, the activity of GRP94 towards proteins and peptides seems to differ significantly from the activities of other molecular chaperones described in this issue. The immunological role of GRP94, which is derived from its peptide binding capacity, is also highly provocative at present. In this review, we examine the current data about GRP94, propose a structural model for the protein and focus on identifying the most interesting experimental questions that the current data pose, such as the mechanism of substrate binding, its selectivity and its regulation.

The GRP94 gene and the regulation of its expression

GRP94 displays an intriguing pattern of evolutionary expression. It is present in vertebrates, some invertebrates like *C. elegans*, and in plants, yet is absent from *Drosophila* and from unicellular organisms like bacteria, archea and yeast. Perhaps this unusual pattern indicates that GRP94 fulfils functions that are mostly necessary in the ER of multicellular organisms.

In every organism with a GRP94 gene, it is a single copy gene. In the human genome the GRP94 gene is on chromosome 12 (q24.2–q24.3), and in addition to the expressed gene there are also two processed pseudogenes.[8] The porcine gene has been sequenced and found to contain 18 exons distributed over 17

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1084-9521/99/050495+11 $30.00/0

kb.[9] Spanning approximately 18 kb, the intron/exon organization of the murine gene is similar, with much higher sequence divergence in the introns than in the coding regions (unpublished observations). All vertebrate GRP94s are extremely conserved,[10] but the sequences of barley and murine GRP94 have diverged considerably, showing only 64% amino acid homology and 49% identity. Nevertheless, they maintain functional homology, since both can associate with murine Ig chains (unpublished observations). The GRP94s genes comprise a distinct subfamily among the HSP90 genes, having diverged from one another early in evolution. Human GRP94 is 62–63% homologous and 46–48% identical at the amino acid level to human HSP90α or HSP90β, a degree of conservation that makes them less similar to one another than the similarity between the trypanosome and human HSP90s.[10] A comparison between the human GRP94 and two cytosolic HSP90s is shown in Figure 1. Apart from a few small deletions and insertions, the degree of homology is fairly constant along the entire sequence. The major differences are in the unique N-terminal and C-terminal extensions of GRP94 that are not found in HSP90α or HSP90β.

While the GRP94 gene is expressed constitutively in all cell types, its expression is up-regulated by a number of stress conditions (ref 11 and references therein). Such conditions include low glucose levels, prolonged anaerobiosis, low extracellular pH, expression of mutated proteins and viral infections. Of note is the lack of induction of mammalian BiP and GRP94 by heat shock, because their promoters lack functional heat shock response elements like those found near many other stress proteins.[12] Laboratory stresses

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hGRP94    MRALWVLGLCCVLLTFGSVRADDEVDVDGTVEEDLGKSREGSRTDDEVVQREEEAIQLDGLNASQIRELREKSEKFAFQA   59
hHSP90                                                                MPEEVHHGEEEVETFAFQA   19

hGRP94    EVNRMMKLIINSLYKNKEIFLRELISNASDALDKIRLISLTDENALSGNEELTVKIKCDKEKNLLHVTDTGVGMTREELV  139
hHSP90    EIAQLMSLIINTFYSNKEIFLRELISNASDALDKIRYESLTDPSKLDSGKELKIDIIPNPQERTLTLVDTGIGMTKADLI   99

hGRP94    KNLGTIAKSGTSEFLNKMTEAQEDGQSTSELIGQFGVGFYSAFLVADKVIVTSKHNNDTQHIWESDSNE.FSVIADPRGN  218
hHSP90    NNLGTIAKSGTKAFMEALQAGADIS.....MIGQFGVGFYSAYLVAEKVVVIRKHNDDEQYAWESSAGGSFTVRAD.HGE  173

hGRP94    TLGRGTTITLVLKEEASDYLELDTIKNLVKKYSQFINFPIYVWSSKTETVEEPMEE.........EEAAKEEKEESDDEAA  290
hHSP90    PIGMGTKVILHLKEDQTEYLEERRVKEVVKKHSQFIGYPITLYLEKEREKEISDDEAEEEKGEKEEEDKDDEEKPKIEDV  253

hGRP94    VEEEEEE....KKPKTKKVEKTVWDWELMNDIKPIWQRPSKEVEEDEYKAFYKSFSKESDDPMAYIHFTAEGEVTFKSIL  366
hHSP90    GSDEEDDSGKDKKKKTKKIKEKYIDQEELNKTKPIWTRNPDDITQEEYGEFYKSLTNDWEDHLAVKHFSVEGQLEFRALL  333

hGRP94    FVPTSAPRGLFDEYGSKKSDYIKLYVRRVFITDDFHDMMPKYLNFVKGVVDSDDLPLNVSRETLQQHKLLKVIRKKLVRK  446
hHSP90    FIPRRAPFDLFEN..KKKKNNIKLYVRRVFIMDSCDELIPEYLNFIRGVVDSEDLPLNISREMLQQSKILKVIRKNIVKK  411

hGRP94    TLDMIKKIADDKYN.DTFWKEFGTNIKLGVIEDHSNRTRLAKLLRFQSSHHPTDITSLDQYVERMKEKQDKIYFMAGSSR  525
hHSP90    CLELFSELAEDKENYKKFYEAFSKNLKLGIHEDSTNRRRLSELLRYHTSQSGDEMTSLSEYVSRMKETQKSIYYITGESK  491

hGRP94    KEAESSPFVERLLKKGYEVIYLTEPVDEYCIQALPEFDGKRFQNVAKEGVKFDESEKTKESREAVEKEFEPLLNWMKDKA  605
hHSP90    EQVANSAFVERVRKRGFEVVYMTEPIDEYCVQQLKEFDGKSLVSVTKEGLELPEDEEEKKKMEESKAKFENLCKLMKEIL  571

hGRP94    LKDKIEKAVVSQRLTESPCALVASQYGWSGNMERIMKAQAYQTGKDISTNYYASQKKTFEINPRHPLIRDMLRRIKEDED  685
hHSP90    D.KKVEKVTISNRLVSSPCCIVTSTYGWTANMERIMKAQAL...RDNSTMGYMMAKKHLEINPDHPIVETLRQKAEADKN  647

hGRP94    DKTVLDLAVVLFETATLRSGYLLPDTKAYGDRIERMLRLSLNIDPDAKVEEEPEEEPEETAED.TTEDTEQDEDEEMDVG  764
hHSP90    DKAVKDLVVLLFETALLSSGFSLEDPQTHSNRIYRMIKLGLG...IDEDE...VAAEEPNAAVPDEIPPLEGDEDASRME  721

hGRP94    TDEEEETAKESTAEKDEL                                                                782
hHSP90    EVD                                                                               724
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Figure 1. Comparison of human GRP94 sequence with that of human HSP90β. The sequences were aligned using the program PileUp and the alignment improved by manual editing: |, amino acid identities; and :, conservative substitutions.

that induce GRP94 expression include treatments with reducing agents, ionophores or tunicamycin, introduction of amino acid analogs and over-expression of transfected proteins.[11] Under all of these conditions GRP94 is up-regulated in parallel with BiP. A feature common to all these stress conditions is that they lead to accumulation of misfolded proteins in the ER, thus the phenomenon was termed the 'unfolded protein response' (UPR, see Gething, this issue).

The UPR has been well characterized at the molecular level in yeast. The essential UPR response element is a 22-bp segment common to the promoters of several UPR responsive ER proteins.[13,14] Accumulation of unfolded protein in the lumen is detected by the ER resident kinase Ire1p,[15,16] perhaps by its ability to measure the amount of free chaperones in the lumen. This eventually leads to the alternative splicing of a transcription factor, Hac1p,[17-19] which recognizes the UPR response element. As ER biogenesis is induced in both higher eukaryotes and yeast by similar physiological demands, it is expected that the signal transduction cascade to the genome would also be similar. Deletion analysis of the rat BiP and GRP94 promoters revealed two common motifs, denoted Core and C1, both of which are responsible for both basal and induced expression.[20,21] Several transcription factors bind to these regions, including YY1 and NF-Y/CBF.[22,23] However, these factors are ubiquitous under normal and stress conditions, and as such, would not be expected to govern stress-specific induction of GRP94. Recently, a basic leucine zipper transcription factor, ATF6, with homology to yeast Hac1p, was found to be involved in the human UPR. It binds to several 19 bp elements (termed ERSE), two of which are within the CORE and C1 regions and are necessary and sufficient for inducing expression of human GRPs.[24]

General features of the protein

The mature murine GRP94 protein consists of 782 residues after cleavage of a 21-residue leader peptide. The protein can be divided into distinct structural domains (Figs. 1 and 2), although their functional significance is only beginning to be elucidated. The N-terminal domain (Figure 2) is highly homologous to that of HSP90, as discussed in more detail below. This domain is followed by an acidic domain, predicted to have a coiled coil conformation, which in GRP94 is shorter than the homologous domain in HSP90. Amino acids 421–448 form a 7-repeat imperfect leucine zipper-like sequence that could serve as a protein–protein interaction module. Another charged domain of unknown function follows (559–609, Figure 2) and it contains a KEKE motif (572–587), which is a proposed protein interaction

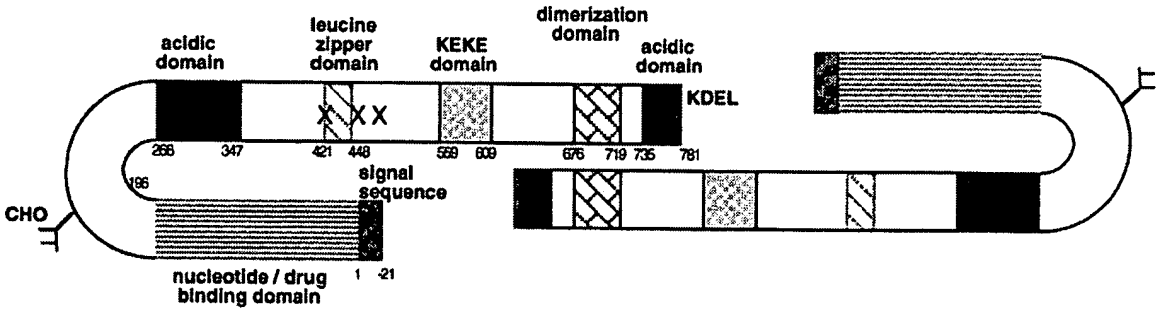

Figure 2. A proposed structural model for GRP94. The protein is depicted as a dimer of candy cane-shaped monomers, associating via the dimerization domain (cross-hatched block) identified in ref 27. The N-terminal signal sequence and the C-terminal KDEL tetrapeptide (checkered blocks) target GRP94 to the ER. The N-terminal domain of the mature protein (horizontal stripes) contains the nucleotide/geldanamycin/ radicicol binding site and the oligosaccharide (CHO) at Asn196. It is followed by the first of two acidic, coiled coil domains (dark blocks) that regulates drug binding.[90] Another charged domain (gray) contains multiple adjacent oppositely charged amino acids and a KEKE protein interaction motif. The shape of the molecule is suggested by EM visualization (refs. in 87) and by the implication of intramolecular masking; the domain containing the three cryptic glycosylation sites (each marked by X) is hyper-glycosylated in mutants made by us (unpublished observations) and by Qu *et al.*[36] The masking may be mediated by both the first acidic domain and the putative leucine zipper domain (diagonal stripes).

motif.[25] The C-terminal 60 amino acids make up a second acidic domain, which is homologous to those found in a number of nuclear proteins and in factors associated with RNA polymerase.

Velocity sedimentation, gel filtration chromatography and rotary shadowing electron microscopy showed that native GRP94 is an elongated, rod-like protein that normally exists almost exclusively as a homo-dimer[26] (Figure 2). Limited proteolysis and *in vitro* translation were used to demonstrate that the C-terminal 23-kDa fragment is both necessary and sufficient for dimerization. A 44-amino acid discrete domain (residues 676–719) from this fragment displayed autonomous dimerization activity.[27] This region contains a hydrophobic stretch (residues 692–709) which is thought to be responsible for subunit association by hydrophobic interactions.[27] Despite similarity in dimerization behavior and homology in the proteolytic cleavage sites between the C-terminal 326 amino acids of GRP94 and the C-terminal 200 amino acids of HSP90α, the two fragments fail to hetero-dimerize *in vitro*. Thus, the interactions driving dimerization *in vivo* must differ between GRP94 and HSP90.[28]

Subcellular localization of GRP94

GRP94 is a resident lumenal protein of the ER: by immunofluorescence and immuno-EM staining it is seen to be uniformly distributed throughout the ER lumen with no preferential association with membrane.[29] GRP94 is also a resident protein of the sarcoplasmic reticulum of muscle cells, a specialized form of ER.[30] Residence in the ER is maintained by the C-terminal tetrapeptide KDEL, which is a target for the KDEL retrieval receptor.[31]

Although normally confined to the ER, GRP94 has been shown to escape the KDEL-mediated retention system in several cell types. For instance, a significant fraction of GRP94 is secreted by hepatocytes, along with other lumenal proteins.[32] In at least one tumor cell line GRP94 is detectable as a surface protein.[33] Likewise, thymocytes of a specific, early developmental stage express 1–5% of their GRP94 and calnexin on the plasma membrane by an unknown mechanism (unpublished observations; ref. 34). Since there is no good evidence for a membrane spanning form of GRP94, it is likely that this ectopic surface expression is mediated by association with other membrane proteins. The significance of surface GRP94 expression is not known.

A remarkable exception to the ER localization of GRP94 is its association with the bile salt-dependent lipase (BSDL), also known as cholesterol esterase. GRP94 in this complex no longer has a KDEL tail.[35] This complex does not dissociate in the ER; rather, it is secreted. The two molecules remain associated from the pancreas to the intestinal lumen, where they can be detected on microvilli. There, the complex is somehow internalized by enterocytes and both molecules are found in an endosomal compartment. BSDL, but not GRP94, is eventually deposited on the basolateral membrane of enterocytes. It is not clear whether GRP94 is needed for BSDL uptake or whether it simply protects BSDL from degradation in the duodenal lumen.[35]

Post-translational modifications

GRP94 is a glycoprotein with six potential Asn glycosylation sites, but only Asn196 is normally used.[36] This Asn is conserved in all known mammalian GRP94 genes. Barley GRP94 lacks this site, but is glycosylated at a novel site at Asn410, between the coiled coil and leucine zipper domains (unpublished observations). Since, under normal circumstances, GRP94 is limited to the ER, its sugar moiety remains as a high-mannose type and is sensitive to endoglycosidase H. A small fraction of GRP94 is hyper-glycosylated under normal expression conditions, and this fractions is increased when GRP94 is over-expressed in either COS or insect cells.[37] *In vitro*, N-terminal deletion mutants of GRP94 are hyper-glycosylated at a cluster of three acceptor sites within a region spanned by residues 424–481 (unpublished observation; ref 36). Glycosylation is apparently unrelated to substrate binding, because de-glycosylated GRP94 can still associate with newly synthesized polypeptides.[38] GRP94 is phosphorylated on Ser and Thr, but not on Tyr residues. The phosphorylation pattern has the hallmarks of casein kinase II action,[30] but the identity of the *in vivo* kinase is not known, as no kinase has yet been rigorously identified in the ER. There is one report of heat-stable, Ca^{2+}-dependent autophosphorylation activity,[39] but this activity has not yet been definitively ascribed to GRP94 by mutational analysis. Another group found that highly purified GRP94 is associated with a heat-labile, Ca^{2+}-inhibited kinase which can be specifically stimulated by BiP.[40] Yet a third report showed that liver GRP94 co-purified with a casein kinase II-like enzyme.[41]

The pattern of phosphorylation of GRP94 is similar

to that of other ER chaperones, such as BiP[42] and calnexin.[43] Using a proteolysis assay, the major sites of casein kinase II-like phosphorylation of canine GRP94 have been localized to a peptide that includes Thr267 and Ser285, and to a C-terminal peptide that starts at Lys733 and contains three Thr residues.[30] Phosphorylation may regulate the activity of GRP94, as phosphorylated GRP94 does not associate with immunoglobulin (Ig) light chain in the cell.[38]

Calcium binding

Like the other abundant lumenal proteins, GRP94 is a low-affinity, high-capacity calcium binding protein, making it one of the important ER calcium buffer proteins. It is believed to have 15 calcium-binding sites, 4 with moderate affinity ($K_D \sim 2$ μM) and 11 with low affinity ($K_D \sim 600$ μM).[3] The calcium binding sites are likely to be in several highly negatively charged regions distributed throughout the GRP94 sequence (Figure 2), as there are no obvious EF hand binding motifs.

In addition to its putative function as a calcium carrier, GRP94 activity may be regulated by calcium binding. *In vitro*, the presence of 100 nM free calcium causes a conformational change, as measured by a decrease in helical content from 40% to 34%.[3] In a non-physiological system, purified GRP94 can bind calmodulin in the presence of calcium, thereby inhibiting the interaction of GRP94 with actin filaments.[44] It is likely that calcium binding also regulates the *in vivo* interactions of GRP94 with other proteins in the ER.

Drug/ATP binding

The N-terminal 35 amino acids of mature GRP94 constitutes a unique sequence with no homology to HSP90 (Figure 1). The sequence 35–274, however, is highly homologous to the fragment 9–236 of human HSP90 and to the fragment 1–220 of yeast HSC82, whose crystal structures have been solved.[45,46] In HSP90, this independently folding domain mediates binding to three structurally unrelated small molecules: ATP, geldanamycin and radicicol. All three bind to the same site, as shown by co-crystallization and by biochemical competition experiments. The binding site is formed by a number of helices that are placed on top of a β sheet platform. In GRP94, all of the residues critical for the formation of this binding site are conserved, and indeed, it binds ATP in a geldanamycin-dependent fashion and has a similar apparent affinity for radicicol as HSP90.[90] The requirements for ATP/drug binding are different between HSP90 and GRP94. Whereas the N-terminal domain of HSP90 is sufficient for binding, the first acidic domain of GRP94 (amino acids 266–347) is also required to obtain equivalent binding.[90] This difference between GRP94 and HSP90 may have important implications for development of drugs that can distinguish between the two proteins. Binding of geldanamycin to GRP94 has been shown to have important functional consequences. The complex between GRP94 and one of its substrates, erbB2 (see below), is disrupted, causing the substrate to be degraded via the ubiquitin/ proteasome pathway.[47,48] Radicicol binding has a similar effect on the fate of another substrate, Ig light chain (unpublished observations).

Association with proteins

A fundamental difference between GRP94 and chaperones like BiP (see Gething, this issue) or calnexin (see Bergeron and Thomas, this issue) is that it interacts with a restricted set of protein substrates. While BiP binds to a large number of proteins that fold in the ER by recognizing a broad range of peptides, and while calnexin recognizes a glycan moiety common to many glycoproteins, GRP94 has so far been shown to associate with relatively few proteins. GRP94 interacts physically with Ig chains,[49] MHC class II,[50] thyroglobulin,[51] erbB2,[47] a herpes virus glycoprotein,[52] apolipoprotein B,[53] collagen,[54] protein C[89] and BSDL.[35] The narrow range of substrate binding is illustrated by the fact that GRP94 has not been detected in association with many other viral glycoproteins nor with MHC class I molecules. Presumably, this restricted substrate specificity is a function of the protein structures that GRP94 recognizes. However, since the list above contains proteins with an Ig fold, a serine protease fold and predominantly helical proteins, no common structural denominator is immediately obvious.

The interaction with Ig light chain is relatively well studied, the preferred substrate being a late, fully disulfide bonded intermediate.[55,56] GRP94 association persists for the majority of the residence time of the light chain in the ER, as opposed to BiP-Ig interactions, which only occur in the first few minutes after Ig synthesis.[55] During the biosynthesis of MHC

class II molecules, GRP94 binds to incompletely assembled intermediates, lacking the invariant chain.[50] In the case of cholesterol esterase, as described above, GRP94 binds tightly to the native protein and traffics with it. Thus, GRP94 seems to associate with advanced folding intermediates of its substrate proteins, or with incompletely assembled oligomers, but not with early folding intermediates. This preference for late folding intermediates is also consistent with the preference of HSP90 for its substrates during *in vitro* refolding reactions.[57-59] The inference from these findings is that through its binding to incompletely folded proteins GRP94 participates in their retention in the ER, like other chaperones, but direct evidence for such a role is lacking.

Another feature of GRP94-protein interactions is that each of the substrate proteins has also been demonstrated to interact with either BiP or calnexin.[50,51,54,55,60] It was not known, therefore, whether GRP94 binding to proteins depends on other molecular chaperones. We have recently shown that GRP94 can bind to the surrogate light chain component VpreB without BiP, while another VpreB population binds BiP and not GRP94 (unpublished observations). The simplest interpretation of these data is that GRP94 does in fact bind directly to substrate proteins.

By analogy to HSP90, it is likely that the function(s) of GRP94 are enhanced by physical interactions with co-chaperones. However, there is currently no direct evidence for the existence of ER homologs of HSP90-associated proteins like p23, Hop, FKBP51 or Cyp40.[61,62]

Taken together, the emerging picture of GRP94 is of a chaperone specific for advanced intermediates in the biosynthesis of proteins, which works downstream of, or in conjunction with, other chaperones. Thus, the ER lumen may contain relay systems of chaperones much like the cytosol of eukaryotic cells or of bacteria, with GRP94 serving as a 'finishing' chaperone in the progression of folding.[56] An important experimental challenge is to define the precise structural features that trigger GRP94 binding, something that has not yet been determined for any protein.

Peptide binding

Is the association of GRP94 with proteins mediated by peptide binding activity? Several lines of evidence suggest that GRP94 does indeed bind peptides. Probably the most compelling are the immunological studies of Srivastava *et al*,[63] Arnold *et al*,[64] and the elution of an antigenic VSV-derived peptide from purified GRP94.[65] In another experimental approach, Lammert *et al* introduced photo-reactive peptides into the cytosol by streptolysin O permeabilization. Following photo-crosslinking, these peptides were found to be associated with only a few ER proteins. The main acceptor was protein disulfide isomerase, but association with GRP94 was also clearly seen.[66] Most of these peptides had properties of peptides translocated via the ER peptide transporter associated with antigen presentation (TAP), including a size distribution of 8–20. Furthermore, when a family of related 9mer peptides was used, in which positions 2 and 9 were systematically mutated, GRP94 displayed a preference for peptides with uncharged amino acids at these positions.[67] These data demonstrate specificity in peptide binding by GRP94, supporting the view that it is a peptide binding protein.

The peptides that bind to GRP94 enter the ER both via the TAP transporter and via a TAP-independent route, suggesting that GRP94 samples the entire ER pool of peptides.[66-68] There is no obvious structural motif common to those peptides that have so far been shown to bind to GRP94. It is noteworthy that even if the average affinity of GRP94 for peptides is tenfold lower than that of MHC class I, the vast excess of GRP94 (and other lumenal peptide binding proteins) would act as a 'sink', significantly decreasing the size of the free pool of peptides in the lumen. Despite speculations in the literature,[63] there is no rigorous evidence that GRP94 physically associates with the TAP complex nor that it is involved in direct peptide loading of class I.[69]

Direct loading of GRP94 *in vitro* with immunologically relevant peptides was also recently demonstrated, although the reaction was inefficient.[70,71] *In vitro* peptide binding of GRP94 results in a conformational change which can be demonstrated by increased binding of hydrophobic dyes, Trp fluorescence and protease sensitivity.[70] These studies do not as yet permit determination of the affinity of GRP94 for peptides, but they now enable the elucidation of the mechanism of peptide binding. It is important to determine why peptide loading of GRP94 is so inefficient compared to chaperones like BiP, what mechanism is used to unload peptides and where the peptide binding site is on GRP94. Perhaps there are other factors involved *in vivo* that facilitate such reactions.

Our current knowledge of the *peptide* binding activity of GRP94 does not yet explain its apparently

small spectrum of *protein* substrates and its preference for advanced folding intermediates. Recent studies with HSP90 proteins showed that they actually have separate peptide binding sites, one at each end of the molecule,[72,73] and that in addition, the C-terminal domain can bind proteins containing a tetratrico peptide repeat (TPR) motif.[74] Perhaps GRP94 is configured similarly, with a peptide-binding site separate from another protein interaction domain.

Nucleotide dependence of substrate binding

A contentious issue in the study of GRP94, as with other HSP90 family members, is the relation between substrate binding and adenine nucleotide binding and hydrolysis. The peptide binding chaperones whose action is understood in molecular detail are all ATPases. GRP94 has also been reported to bind immobilized ATP[75,76] and to cleave ATP.[77] However, the inherent ATPase activity of GRP94 preparations is much slower than even the sluggish activity of BiP and the ATP binding activity is at least 20-fold weaker than that of BiP (ref. 76; unpublished observations). Furthermore, *in vitro* peptide binding by GRP94 is ATP-independent[78] and the *in vivo* association with immunoglobulin chains is maintained when cellular ATP is depleted.[49] Thus, the role of adenine nucleotides in regulating GRP94 activity is still unclear.

GRP94 and antigen presentation

The peptide binding activity of GRP94 makes it, in principle, a highly attractive natural immunological vaccine. GRP94 received much recent attention as a potential cancer vaccine, because tumor cells commonly develop multiple ways to evade discovery by the immune system. When injected into syngeneic mice, purified GRP94 from the murine sarcomas meth A or CMS5 induced protection against a subsequent injection of the tumor from which it was derived, but not against a challenge with a different tumor.[7] Furthermore, injection of tumor-derived GRP94 led to a T cell response that rejected an already established tumor.[79] It was later found that GRP94 itself was not mutated in the tumors, indicating that a bound peptide stimulated protective immunity, rather than GRP94 *per se*.[80] As described above, acid elution of purified GRP94 indeed yields a heterogeneous mixture of peptides, including some capable of eliciting the relevant T cell responses.[65,77] Treatments designed to dissociate the binding, such as partial unfolding with heat or guanidine hydrochloride, abolish the antigenicity of GRP94.[81] Recently, complexes of either HSP70 or GRP94 and a variety of peptides were reconstituted *in vitro* and found to be immunologically active. Upon injection into mice, they were able to elicit anti-tumor immunity and specific $CD8^+$ cytolytic T lymphocyte response in the same manner as gp96-peptides complexes isolated from tumors.[71]

GRP94 does not trigger T cells directly; rather, generation of the response requires antigen presenting cells, like macrophages or dendritic cells, that express the appropriate MHC class I on their surface.[63] The responding cells are cytotoxic T cells whose receptors can only contact peptides when they are bound in the grooves of specific alleles of MHC class I molecules (Figure 3). Thus, to elicit a T cell response, GRP94 must somehow transfer the peptide to the appropriate MHC class I. Presumably, GRP94-peptide complexes are taken up by endocytosis into the antigen presenting cells, perhaps via specific receptors (Figure 3). Direct demonstration of specific GRP94 uptake has recently been provided,[91,92] however, the nature of the intracellular uptake route has not yet been fully delineated, nor is it known whether only a specific subset of cells is endowed with the ability to funnel GRP94-bound peptides into the presentation pathway. This information is critical for the design of optimal immunization protocols and for the potential use of GRP94 in conjunction with cytokines to boost antigen presenting cells in cancer patients.

Another unresolved issue is the compartment in which the peptides are loaded onto class I molecules. A pathway for the induction of exogenous peptides to the ER was demonstrated by Day *et al.*[82] It seems likely that the peptides are loaded onto class I molecules in the ER, rather than in another compartment along the secretory or endocytic pathways, given that peptides stabilize the quaternary structure of class I and that 'empty' class I molecules are deficient in their intracellular transport.[83] Furthermore, several peptides which were shown to be presented via the GRP94-assisted pathway were also found to be imported into the ER by the TAP transporter[66] that associates with nascent class I molecules in the ER.[84] Thus, it is likely that the loading of exogenous peptides occurs in the ER (Figure 3), which is the normal compartment for class I loading. Nonetheless, an important unanswered question is whether GRP94 escorts its bound peptides all the way from the cell's

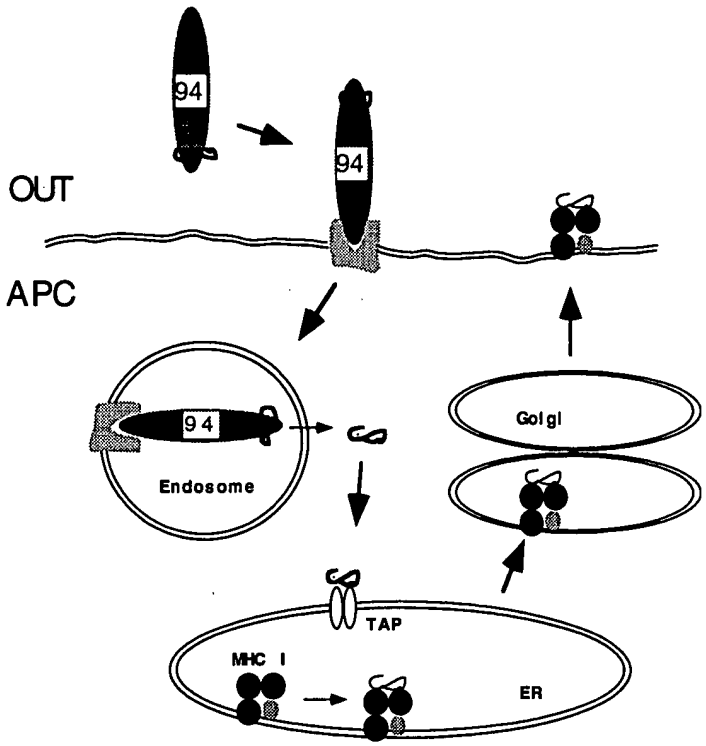

Figure 3. The re-presentation hypothesis for GRP94's role in immune responses. Extracellular GRP94 loaded with peptide is envisioned as released from dying cells, either during viral infection of a tissue or necrosis of cells within solid tumors. This protein could be recognized by a surface protein (grey) on professional antigen presenting cells, like tissue macrophages, which acts as a GRP94 receptor. GRP94-peptide complexes are internalized into an undefined endocytic compartment, where GRP94, which is sensitive to proteolysis, is degraded. The peptide thus released is translocated to the cytosol via pathways known to exist in endocytic organelles[88]. From this point onward, the normal pathway for endogenous peptide presentation is utilized. The GRP94-derived peptide is imported into the ER via the TAP transporter and loaded onto newly synthesized MHC class I molecules. These molecules then follow the normal exocytic route to the cell surface and present the peptide to cytotoxic T cells.

exterior to the ER, or whether it unloads the peptides in endosomes or in the cytosol. The answers to these questions will go a long way towards enabling the medical use of the GRP94 presentation pathway.

It is worth noting that the ability to augment antigen presentation to T cells is not unique to GRP94. Proteins from the HSP70 family (from the cytosol as well as the ER) are also endowed with this capacity,[85,86] as is calreticulin. However, there is specificity in this pathway, since different peptide binding chaperones differ in their efficacy in exogenous peptide presentation, and the differences are not a simple function of their binding capabilities.[86] Thus, calreticulin is as effective as GRP94 in this pathway of peptide presentation, whereas protein disulfide isomerase, which in the system described in ref. 66 is the major peptide binding protein, has not been reported to be effective in peptide presentation.

Conclusions

The immunological use of GRP94 as a tumor vaccine illustrates the promise of harnessing the peptide binding capacity of this molecule for innovative medical applications. This promise in turn highlights the need to solve the enigmatic problems regarding the mechanism and selectivity of substrate binding by GRP94, and the regulation of its activity *in vitro* and *in vivo*. It is likely that as answers to these questions illuminate GRP94's physiological roles in the ER, they will also suggest effective strategies for its medical use as a peptide (or protein) delivery system.

Acknowledgements

We thank the members of the Argon laboratory for the unpublished observations cited in this paper. This work was supported by grants from NIH and the American Cancer Society.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 271, No. 9, Issue of March 1, pp. 4974–4977, 1996
Printed in U.S.A.

p185^{erbB2} Binds to GRP94 *in Vivo*

DISSOCIATION OF THE p185^{erbB2}/GRP94 HETEROCOMPLEX BY BENZOQUINONE ANSAMYCINS PRECEDES DEPLETION OF p185^{erbB2}*

(Received for publication, November 7, 1995)

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Treatment of SKBr3 cells with benzoquinone ansamycins, such as geldanamycin (GA), depletes p185^{erbB2}, the receptor tyrosine kinase encoded by the *erbB2* gene. In the same cells, a biologically active benzoquinone photoaffinity label specifically binds a protein of about 100 kDa, and the ability of various GA derivatives to reduce the intracellular level of p185^{erbB2} correlates with their ability to compete with the photoaffinity label for binding to this protein. In this report, we present evidence that the ~100-kDa ansamycin-binding protein is GRP94. Membrane-associated p185^{erbB2} exists in a stable complex with GRP94. GA binding to GRP94 disrupts this complex, leading to degradation of pre-existing p185^{erbB2} protein, and resulting in an altered subcellular distribution of newly synthesized p185^{erbB2}.

Herbimycin A (HA)[1] and geldanamycin (GA) are benzoquinone ansamycins with potent antiproliferative activity that specifically bind to the heat shock protein hsp90 (1), with which several tyrosine kinases, as well as other intracellular signal transduction molecules, are complexed (2–9). Geldanamycin dissociates certain multi-molecular complexes containing HSP90 (1, 10), leading to target protein (*i.e.* v-Src, c-Raf-1) instability (1, 9).

The *erbB2* gene (also known as *her-2/neu*) encodes a 185-kDa receptor-like protein (p185^{erbB2}) with tyrosine kinase activity. This protein is overexpressed in many breast, ovarian, and prostate carcinomas and is associated with poor prognosis. Miller *et al.* (11) have reported that p185^{erbB2} is rapidly depleted in human breast cancer cells (SKBr3) following exposure to HA or GA. Although the p185^{erbB2} protein level is markedly reduced, p185^{erbB2} mRNA and protein synthesis are only slightly affected, and it appears that one of the primary effects of these drugs is to significantly reduce the half-life of p185^{erbB2} (11, 12). However, unlike v-Src and c-Raf-1 (see above), p185^{erbB2} cannot be demonstrated to form a complex with HSP90 (12). Intriguingly, when they used a biologically active, ^{125}I-labeled photoaffinity derivative of GA (CP202509), Miller *et al.* (13) demonstrated specific binding to a 100-kDa protein in SKBr3 cells, but not to p185^{erbB2} itself. The ability of various GA derivatives to reduce the intracellular level of p185^{erbB2} correlated with their ability to specifically compete with the ^{125}I-photoaffinity label for binding to this protein.

In this report, we present evidence that this ansamycin-binding protein is the glucose-regulated protein GRP94, an endoplasmic/sarcoplasmic reticulum protein with homology to the molecular chaperone HSP90 (14–16). An abundant cellular glycoprotein, GRP94 is induced in response to glucose deprivation, hypoxia, calcium ionophores, glycosylation inhibitors, and low pH, and its expression is up-regulated in pathological states during which these conditions naturally occur, such as ischemia and tumor growth (reviewed in Ref. 17). While the function of GRP94 has not been well defined, it has been implicated in Ca^{2+} regulation (18), protein folding, and antigen presentation (19, 20). In addition, we demonstrate here the existence of a stable heterocomplex between p185^{erbB2} and GRP94. Finally, we show that GA rapidly dissociates GRP94 from p185^{erbB2} and that this occurs prior to degradation of p185^{erbB2} protein.

MATERIALS AND METHODS

Cell Culture Conditions—SKBr3 human breast carcinoma cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals), 1 mM glutamine, and 10 mM Hepes (pH 7.3), at 37 °C in an atmosphere of 6% carbon dioxide, or as described previously (13).

Preparation of Cytosol and Crude Membrane Fractions from SKBr3 Cells—10^7 SKBr3 cells were plated in 100-mm^2 plastic dishes. Following treatment, cells were washed twice with ice-cold PBS and scraped with a cell scraper into 1 ml of ice-cold TESV buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1 mM vanadate) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 20 μg/ml each aprotinin and leupeptin). Cells were homogenized using a Dounce tissue grinder and then lysed by sonication on ice for 10 s three times, letting samples chill in ice water between sonications. The cell lysate was ultracentrifuged at high speed (100,000 × *g*, 60 min, 4 °C) for preparation of membrane and cytosol fractions, or at low speed (12,000 × *g*, 15 min, 4 °C) to remove the insoluble fraction. The supernatant fraction from high speed centrifugation (cytosol) was saved, while the crude membrane pellet was resuspended by sonication in 0.5 ml of ice-cold TESV buffer containing protease inhibitors. The membrane suspension was then clarified by centrifugation at 12,000 × *g* for 15 min (4 °C), and the supernatant (soluble membrane fraction) was saved. The protein content of each fraction was determined using the BCA protein assay (Pierce).

Co-immunoprecipitation of GRP94 and p185^{erbB2} and Western Immunoblot—GRP94 protein was immunoprecipitated from both membrane and cytosol fractions (500–1000 μg of protein, prepared fresh) using 2 μg of rat monoclonal anti-human GRP94 antibody (StressGen) with an incubation period of 1 h at 4 °C. Rabbit anti-rat protein A-Sepharose beads, preswollen and equilibrated in TESV, were added, and samples were rotated in a tumbler for 40 min at 4 °C. The beads were washed three times with TESV buffer, resuspended in 1 × sample loading buffer (0.0627 M Tris-HCl, pH 6.8, 1% SDS, 1% 2-mercaptoeth-

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[1] The abbreviations used are: HA, herbimycin A; GA, geldanamycin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

anol, 10% glycerol, 0.0005% bromphenol blue), and heated for 5 min at 100 °C. Samples were then electrophoresed through a 7% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. $p185^{erbB2}$ was detected with a monoclonal antibody (Ab3, 1:2,000 dilution, Oncogene Science) using a chemiluminescence-based Western blotting kit (DuPont) according to supplier's instructions. Membranes were exposed for various times to Kodak X-Omat AR film. Films were scanned by densitometry to obtain semi-quantitative analysis of $p185^{erbB2}$ level. Alternatively, the whole cell lysate, cleared of insoluble particles after sonication, was immunoprecipitated with 2 μg of a monoclonal antibody targeted against human $p185^{erbB2}$ (Ab 5, Oncogene Science). Detection of GRP94 was performed using a 1:1,000 dilution of the StressGen monoclonal antibody, as described above. The content of GRP94 and $p185^{erbB2}$ in cytosol and crude membrane fractions was monitored by immunoblotting 100 μg of cytosol and 15 μg of membrane protein with the proper monoclonal antibody, as described above.

^{125}I-CP202509 Labeling of SKBr3 Cells—Two million cells were seeded in 60-mm plates. After overnight culture, cell monolayers were incubated with 11 nM ^{125}I-CP202509 (15 μCi) for 1 h at 37 °C in the dark in the presence or absence of 5 μM unlabeled CP127374, a geldanamycin analog. After aspiration of the medium containing compound and addition of 1 ml of PBS, the cells were irradiated for 10 min with a UV light (λ = 254 nm) at a distance of 8–10 cm. The monolayers were washed twice with PBS and lysed with hot 2% SDS, 50 mM Tris, pH 7.4. Lysates were boiled for 10 min and pulse-sonicated for 1 min in a bath sonicator (Heat Systems Inc., Farmingdale, NY).

Immunoprecipitation of ^{125}I-CP202509-labeled Ansamycin-binding Protein—Denatured lysates of SKBr3 cells were first diluted 20-fold in PBS containing 0.25% bovine serum albumin to reduce the SDS concentration to a level suitable for the formation of antigen-antibody complexes. Diluted lysates were incubated on ice for 10 min and centrifuged at 100,000 × *g* for 30 min. Lysate supernatants were incubated with goat anti-rat conjugated agarose beads (Sigma), which had been coated with rat monoclonal antibody to either GRP94 or HSP90 (StressGen, Vancouver, British Columbia, Canada). The bead-lysate mixtures were incubated for 2 h at 4 °C. on a rocker. Finally, beads were washed six times with wash buffer (50 mM Tris, pH 7.4, 0.1% SDS) and boiled for 5 min with 2 × Laemmli sample buffer (120 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 100 mM dithiothreitol, 0.016% bromphenol blue). Eluted proteins were analyzed by PAGE and autoradiography.

To verify the specificity of the immunoprecipitation, lysates of unlabeled SKBr3 cells were prepared in parallel with the above samples. Immunoprecipitation and immunoblotting with both antibodies revealed that neither cross-reacted with the other's target (native or denatured), demonstrating that both antibodies, whether used for immunoprecipitation or immunoblotting, are specific for their cognate antigens.

Subcellular Localization of Synthesized $p185^{erbB2}$ in the Presence or Absence of GA—Subcellular localization of $p185^{erbB2}$ protein in untreated SKBr3 cells, and in cells exposed for 18 h to 2 μM GA, was visualized by immunofluorescence. Briefly, cells were grown on coverslips, rinsed in PBS, fixed with 3.7% formaldehyde for 10 min at room temperature, and rinsed again with PBS. Coverslips were overlaid with $p185^{erbB2}$ antibody (Ab 5, 10 μg/ml in PBS, Oncogene Science) and kept at 4 °C for 1 h. Following rinsing in PBS, coverslips were overlaid with Cy3TM-conjugated goat anti-mouse immunoglobulin (1:500 in PBS; Jackson ImmunoResearch Laboratories, Inc.) and kept at 4 °C for an additional hour. After rinsing in PBS and water, coverslips were air-dried and mounted with SlowFade® mounting medium (Molecular Probes). Fluorescence was visualized using a Zeiss Axioskop microscope and an Optronics CCD camera.

Analysis of Newly Synthesized $p185^{erbB2}$ in the Presence or Absence of GA—A total of 10^7 SKBr3 cells were plated in 100-mm^2 plastic dishes and allowed to grow for 24 h. Cells were exposed to medium alone or containing GA for 16 h, then washed twice with ice-cold PBS and incubated for an additional 6 h in 4 ml of Dulbecco's modified Eagle's medium lacking methionine and cysteine, supplemented with 10% dialyzed fetal calf serum and 100 μCi/ml [^{35}S]methionine/cysteine (Tran^{35}S-label, ICN). GA (3 μM) was added back to cells previously exposed to the drug. At the end of the labeling period, cells were washed twice in cold PBS and lysed as described above. Protein content was determined, as well as the amount of radioactivity in 5 μl of cell lysate that was precipitable with trichloroacetic acid. $p185^{erbB2}$ was immunoprecipitated from lysates containing equal acid-precipitable radioactivity (Ab 5, 2 μg/sample, Oncogene Science) as described above. Immunoprecipitates were solubilized by heating at 95 °C for 5 min in 1% SDS. After centrifugation, supernatants were transferred to tubes containing an equal volume of digestion buffer (0.1 M sodium acetate, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pH 5.5) and incubated with endoglycosidase H (Boehringer Mannheim, 10 milliunits/tube) for 20 h at 37 °C. Samples were then mixed with 5 × reducing loading buffer and electrophoresed through a 7% SDS-polyacrylamide gel, which was then fixed in 50% methanol, 10% acetic acid, and the [^{35}S] signal was amplified using an enhancing solution (DuPont). Dried gels were autoradiographed using Kodak X-Omat AR films.

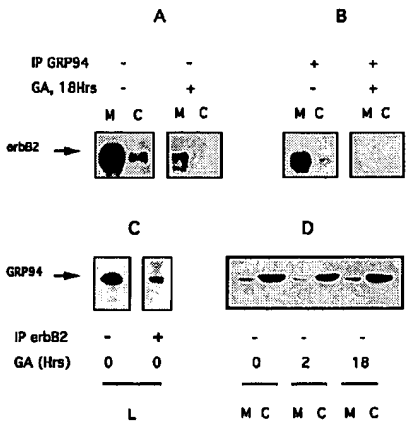

FIG. 1. **$p185^{erbB2}$ and GRP94 exist as a complex in SKBr3 cells.** SKBr3 cells were incubated 18 h in the presence (+) or absence (−) of GA (3 μM). Prior to SDS-PAGE analysis, proteins contained in membrane (*M*) or cytosol (*C*) fractions were (*panel B*) or were not (*panels A* and *D*) immunoprecipitated with rat monoclonal anti-human GRP94. In *panel C*, proteins in total lysate (*L*) were (+) or were not (−) subjected to immunoprecipitation with monoclonal anti-human $p185^{erbB2}$. The proteins were separated by electrophoresis and immunoblotted with monoclonal antibodies to $p185^{erbB2}$ (*panels A* and *B*) or to GRP94 (*panels C* and *D*) as described under "Materials and Methods."

RESULTS

$p185^{erbB2}$ and GRP94 Form an Intracellular Complex in SKBr3 Cells Which Is Dissociated by Brief Exposure to GA—After overnight incubation of SKBr3 cells in the presence or absence of GA, membrane and cytosol fractions were prepared. Detection of a native complex between $p185^{erbB2}$ and GRP94 was demonstrated by co-precipitation of $p185^{erbB2}$ with a monoclonal antibody targeted against human GRP94 (Fig. 1*B*). The native complex was easily seen in the membrane fraction, which contained the majority of $p185^{erbB2}$, but a minority of GRP94. In most experiments, a $p185^{erbB2}$·GRP94 complex could also be demonstrated in cytosol, but at a much lower level (for example, see Fig. 1*B*). The presence of a GRP94·$p185^{erbB2}$ complex was confirmed by the converse experiment, *i.e.* immunoprecipitation of total lysate with antibody to $p185^{erbB2}$ followed by Western blot detection of GRP94 (Fig. 1*C*). After overnight treatment with GA, the complex disappeared from both cellular fractions (see Fig. 1*B*). However, such treatment caused the level of $p185^{erbB2}$, but not GRP94, to be dramatically reduced (Fig. 1, compare panels *A* and *D*).

In order to determine whether disappearance of the $p185^{erbB2}$·GRP94 complex following overnight exposure to GA was the result of loss of $p185^{erbB2}$, or the result of complex dissociation prior to loss of $p185^{erbB2}$, we performed the same experiment following a brief exposure of SKBr3 cells to GA. As seen in Fig. 2, after a 75-min exposure of SKBr3 cells to GA, total $p185^{erbB2}$, as measured by Western blotting of cell lysates, was only minimally reduced (see Fig. 2*A*). However, co-precipitation of a $p185^{erbB2}$·GRP94 complex was dramatically reduced (see Fig. 2*B*).

Benzoquinone Ansamycins Bind Directly to GRP94—When intact SKBr3 cells were labeled with ^{125}I-CP202509, a GA analog incorporating a photoactivatable linker, a ~100-kDa protein in the total lysate was specifically labeled in a photolysis-dependent manner (Fig. 3, compare *lanes 1* and *2*), as

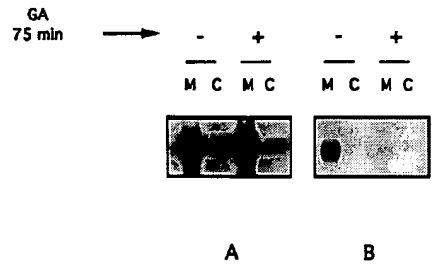

FIG. 2. **The native complex between $p185^{erbB2}$ and GRP94 is dissociated by GA prior to $p185^{erbB2}$ depletion.** SKBr3 cells were incubated for 75 min in the presence (+) or absence (−) of GA (3 μM). Prior to SDS-PAGE analysis, proteins contained in membrane (*M*) or cytosol (*C*) fractions were (*panel B*) or were not (*panel A*) immunoprecipitated with rat anti-human GRP94. Immunoblotting of $p185^{erbB2}$ was performed using a murine anti-human $p185^{erbB2}$ antibody.

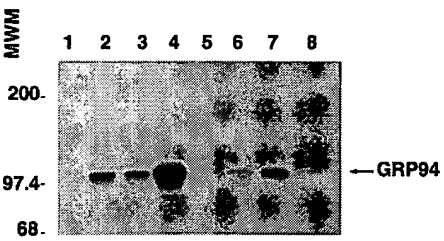

FIG. 3. **GA binds to cellular GRP94.** SKBr3 cells were labeled with ^{125}I-CP202509 and lysed as described under "Materials and Methods." Lysates were subjected to immunoprecipitation with monoclonal antibodies to GRP94 or HSP90 as described. The positions of protein molecular size standards are indicated (in kDa). *Lane 1*, lysate of cells incubated with ^{125}I-CP202509 but not exposed to UV light. *Lane 2*, lysate of cells incubated with ^{125}I-CP202509 followed by photolysis. *Lane 3*, lysate in *lane 2* after clearing by immunoprecipitation with anti-GRP94. *Lane 4*, anti-GRP94 immunoprecipitate from lysate in *lane 2*. *Lane 5*, lysate of cells incubated with ^{125}I-CP202509 and 5 μM CP127374 followed by photolysis. *Lane 6*, anti-GRP94 immunoprecipitate from lysate in *lane 5*. *Lane 7*, lysate in *lane 2* after clearing with anti-HSP90. *Lane 8*, anti-HSP90 immunoprecipitate of lysate in *lane 2*.

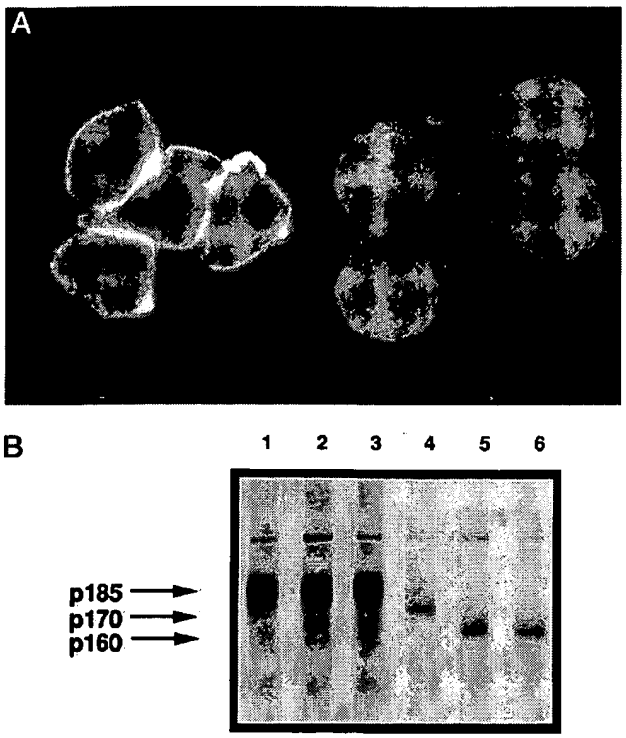

FIG. 4. **GA alters the subcellular distribution of newly synthesized $p185^{erbB2}$.** *A*, cells, grown on coverslips, were treated with GA (2 uM for 22 h), and then $p185^{erbB2}$ immunofluorescence was detected as described under "Materials and Methods." Results are compared to untreated cells. *B*, cells treated as in *A* were pulse-labeled with [^{35}S]methionine/cysteine for the final 6 h. $p185^{erbB2}$ was immunoprecipitated from total cell lysate and subjected to overnight digestion with endoglycosidase H as described under "Materials and Methods." *Lanes 1–3* represent $p185^{erbB2}$ immunoprecipitations from control cells, and *lanes 4–6* represent drug-treated cells. *Lanes 1* and *4*, no endoglycosidase H; *lanes 2* and *5*, 10 milliunits of endoglycosidase H; *lanes 3* and *6*, 20 milliunits of endoglycosidase H.

reported previously (13). After GRP94 immunoprecipitation, the labeled ansamycin-binding protein was reduced in the cleared lysate (Fig. 3, compare *lane 3* to *lane 2*), and concentrated in the GRP94 immunoprecipitate (Fig. 3, *lane 4*). Labeling of GRP94 by ^{125}I-CP202509 was specific and saturable, because co-incubation of the cells with 5 μM unlabeled CP127374 markedly reduced labeling of the ~100-kDa protein in both total lysate (Fig. 3, compare *lane 5* to *lane 2*) and in the GRP94 immunoprecipitate (Fig. 3, compare *lane 6* to *lane 4*). In contrast, the ^{125}I-CP202509-labeled protein was not immunoprecipitated with anti-HSP90 (Fig. 3, *lanes 7* and *8*). Photoaffinity labeling of 10% Triton X-100 extracts of SKBr3 cells with ^{125}I-CP202509 also resulted in specific labeling of a ~100-kDa protein, which was immunoprecipitated by anti-GRP94 antibody, confirming the results obtained with intact cells (data not shown). Superimposition of an autoradiogram and GRP94 immunoblot from a single transfer membrane indicated that the ^{125}I-CP202509-labeled band and GRP94 had identical electrophoretic mobility. Thus, these data identify the target of the GA photoaffinity analog, previously described as p100, as GRP94, on the basis of both molecular weight and immunoprecipitation with an anti-GRP94 monoclonal antibody.

Prolonged Exposure to GA Modifies the Subcellular Distribution of $p185^{erbB2}$—Approximately 85% of $p185^{erbB2}$ is associated with the membrane fraction of SKBr3 cells (based on densitometric analysis of data in Fig. 1*A* and similar experiments). Since GA treatment greatly depletes the steady-state level of $p185^{erbB2}$ without affecting its synthesis (11, 12), we wished to determine whether prolonged exposure to GA altered the subcellular distribution of newly synthesized protein. To do this, we analyzed $p185^{erbB2}$ immunofluorescence in intact cells, both untreated or exposed to GA for 22 h. As can be seen in Fig. 4, in untreated SKBr3 cells $p185^{erbB2}$ is primarily localized to the plasma membrane. This corresponds to the expected strong signal seen by PAGE analysis of the membrane fraction (see Fig. 1*A*).

After 22 h in the presence of GA, however, immunofluorescence analysis revealed that $p185^{erbB2}$ was no longer localized to the plasma membrane, but instead appeared restricted to cytoplasmic inclusions, as visualized by a punctate pattern of perinuclear fluorescence (Fig. 4*A*). Since GA depletes pre-existing $p185^{erbB2}$ protein within several hours (11), and reduces the half-life of newly synthesized protein from greater than 9 h to approximately 2 h (12), the immunofluorescent signal observed in Fig. 4*A* most likely represents $p185^{erbB2}$ newly synthesized in the presence of GA. Immunoprecipitation and PAGE analysis of newly synthesized $p185^{erbB2}$ (using a 6-h pulse with [^{35}S]methionine) from cells treated with GA for 24 h revealed that the ratio of distribution of the protein between membrane and cytosol remained the same as in untreated cells (83% of total signal in membrane fraction; data not shown). However, drug treatment rendered all of the newly synthesized material sensitive to endoglycosidase H, while $p185^{erbB2}$ synthesized in the absence of GA was only slightly sensitive to endoglycosdiase H (Fig. 4*B*). Endoglycosidase H sensitivity is characteristic of incomplete glycosylation and is consistent with the lower apparent molecular weight of $p185^{erbB2}$ synthesized in

the presence of GA (Fig. 4*B*, compare *lanes 1* and *4*). Since resistance to endoglycosidase H is acquired in the trans-Golgi (21), these data are further consistent with the trapping of $p185^{erbB2}$ synthesized in the presence of GA in the endoplasmic reticulum or cis-Golgi. Thus, although overnight exposure to GA does not change the fraction of protein that is membrane associated, it causes dramatic subcellular redistribution of newly synthesized $p185^{erbB2}$.

DISCUSSION

In this report, we demonstrate that the $p185^{erbB2}$ receptor tyrosine kinase forms a stable complex with the glucose-regulated chaperone protein GRP94. The benzoquinone ansamycin GA destabilizes this complex within 75 min, prior to significant loss of $p185^{erbB2}$, which occurs over the next several hours. Exposure of SKBr3 cells to GA and other benzoquinoid ansamycins has been shown previously to result in rapid loss of $p185^{erbB2}$ (11). This effect appears to be mediated primarily at the level of protein stability, since $p185^{erbB2}$ mRNA level and rate of synthesis remain essentially unaltered, while the half-life of the protein is reduced from 9.5 to 2 h (11, 12). Effects of benzoquinone ansamycins on other receptor tyrosine kinases have been noted. Thus, Murakami *et al.* (22, 23) reported destabilizing effects of HA on the epidermal growth factor receptor, while Sepp-Lorenzino *et al.* (24) described similar effects of HA on both the insulin receptor and the insulin-like growth factor receptor. Sepp-Lorenzino *et al.* (24) suggest that the protein instability caused by HA is mediated by the 20 S proteasome in a ubiquitin-dependent manner.

Previously, using an iodinated photoaffinity analog of GA, Miller *et al.* (13) demonstrated that this compound did not label $p185^{erbB2}$, but instead bound an unknown protein of approximately 100 kDa. Furthermore, the ability of various GA analogs to deplete cellular $p185^{erbB2}$ correlated with their ability to interact with p100 (13). The data presented here identify p100 as GRP94, which can be co-precipitated in a complex with $p185^{erbB2}$. By binding to GRP94, GA either induces rapid dissociation of this complex or interferes with the dynamic equilibrium of complex association-dissociation. This occurs prior to significant loss of $p185^{erbB2}$, suggesting a role for GRP94 in maintaining $p185^{erbB2}$ stability. Whether ansamycin disruption of GRP94-$p185^{erbB2}$ complexes leads to ubiquitination of $p185^{erbB2}$ and its subsequent proteolysis by the 20 S proteasome is currently under investigation.

Other receptors may be regulated in a similar fashion. For example, the type I tumor necrosis factor receptor, which has no tyrosine kinase activity, has recently been shown to form a native complex, whose function is as yet undetermined, with a protein showing strong homology to members of the HSP90 family (25).

Our current data further suggest that failure of newly synthesized $p185^{erbB2}$ to associate with GRP94 prevents the translocation of the newly synthesized protein to the plasma membrane, instead trapping it in an intracellular vesicular compartment consistent with the endoplasmic reticulum/cis-Golgi. The data in Fig. 4 demonstrate that, following overnight exposure to GA, $p185^{erbB2}$ is only detectable in this vesicular compartment and not on the plasma membrane. Perhaps an association with GRP94 is required for both the proper intracellular trafficking and stability of a family of receptor proteins.

GA has previously been shown to bind to the heat shock protein HSP90, with which GRP94 shares 50% homology (26). The drug causes dissociation of heterocomplexes composed of this protein and various signal transduction proteins, including v-Src, c-Raf-1, and the progesterone receptor (1, 9, 10). In the case of c-Raf-1 and v-Src, heterocomplex dissociation results in protein instability and altered subcellular localization (1, 9, 27). These findings are thus quite similar to those reported here, except $p185^{erbB2}$ apparently associates with GRP94 and not HSP90. It is not clear why the GA photoaffinity label only recognizes GRP94 and not HSP90 in either intact cells or cell lysates. We have documented that SKBr3 cells contain normal amounts of HSP90, and that this HSP90 efficiently binds to solid phase-immobilized GA in a competable manner (1).[2] In addition, the photoaffinity label binds effectively to purified HSP90 in a photolysis-dependent manner *in vitro*.[2] Conversely, GRP94 binds to solid phase-immobilized GA, corroborating the photoaffinity label results, but GRP94 binding is less efficient than that of HSP90.[2] A possible explanation for these apparent discrepancies is that GA and its photoaffinity label derivative, although both effective in depleting $p185^{erbB2}$ (13), possess different affinities for GRP94 and HSP90. At the same time, substitution with a bulky group at the 17-position of GA (necessary for solid phase immobilization of GA; see Ref. 1) may decrease the drug's binding affinity to GRP94 without affecting binding to HSP90. In fact, certain 17-substituted GA derivatives are poor depletors of $p185^{erbB2}$ (28),[2] presumably because of their failure to interact with GRP94.

The current results, together with previous reports of GA effects on HSP90 (1, 10), identify the molecular site of action of the benzoquinone ansamycins as the chaperonins, specifically HSP90 and GRP94. Binding of these inhibitors to the chaperonins appears to destabilize the complex of an array of signaling proteins, such as $p185^{erbB2}$, v-Src, or c-Raf-1, with the chaperone protein, thereby directing the signaling protein to a degradative pathway. Further studies should better define the role of these chaperones in regulating signal transduction as well as the potential of the benzoquinone ansamycins in the pharmacologic manipulation of this process.

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Immunization with a HER-2/neu h lper p ptide vaccine generates HER-2/neu CD8 T-c ll immunity in cancer patients

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Received for publication November 10, 2000, and accepted in revised form January 3, 2001.

CD4 T-cell help is required during the generation and maintenance of effective antitumor CD8 T cell–mediated immunity. The goal of this study was to determine whether HER-2/neu–specific CD8 T-cell immunity could be elicited using HER-2/neu–derived MHC class II "helper" peptides, which contain encompassed HLA-A2–binding motifs. Nineteen HLA-A2 patients with HER-2/neu–overexpressing cancers received a vaccine preparation consisting of putative HER-2/neu helper peptides p369–384, p688–703, and p971–984. Contained within these sequences are the HLA-A2–binding motifs p369–377, p689–697, and p971–979. After vaccination, the mean peptide-specific T-cell precursor frequency to the HLA-A2 peptides increased in the majority of patients. In addition, the peptide-specific T cells were able to lyse tumors. The responses were long-lived and detectable for more than 1 year after the final vaccination in select patients. These results demonstrate that HER-2/neu MHC class II epitopes containing encompassed MHC class I epitopes are able to induce long-lasting HER-2–specific IFN-γ–producing CD8 T cells.

J. Clin. Invest. **107**:477–484 (2001).

Introduction

The cytolytic CD8 T cell is generally thought to be the major mediator of antitumor immunity. Many tumor antigens have been discovered within the last decade, from which multiple MHC class I–restricted epitopes have been identified (1). One such tumor antigen that is overexpressed on several cancers, including breast and ovarian cancers, is HER-2/neu, the gene product of *erbB2/neu* proto-oncogene (2–4). Using both predictive and MHC elution techniques, it has been possible to identify several MHC class I–restricted epitopes of HER-2/neu for use in immune-based cancer therapies (5–7).

Antitumor immunization strategies have taken many forms, including the use of whole cell-, peptide-, protein-, and DNA-based vaccines. Peptide-based vaccines are attractive over other forms because peptides are (a) easily constructed, (b) chemically stable, (c) free of contaminating substances such as bacterial pathogens, and (d) devoid of oncogenic potential. Although some successful immunization has been achieved using MHC class I–restricted peptide-based vaccines (8, 9), many studies with native, unmodified peptide resulted in no response or only low-level responses (10–13). For example, Pass and colleagues demonstrated generation of peptide-specific precursors to the $gp100_{209-217}$ in five of six melanoma patients following immunization (8). In contrast, in parallel studies from the same group it was observed that only two of seven and zero of seven patients had detectable precursors to $gp100_{280-288}$ or MART-1_{27-35}, respectively, after immunization (8). Immunity to MHC class I peptides can be augmented by adding "help" in the form of CD4 T-helper cells. CD4 T-cell responses are essential, both in order to extend the life of the antitumor CD8 T cells and to promote the accumulation of antigen-presenting cells at the tumor site (14).

The necessity of CD4 T-cell help to generate and sustain the MHC class I–restricted CD8 T-cell responses has led to the use of universal, nonspecific MHC class II–restricted epitopes such as PADRE in clinical vaccination trials (15, 16). Although responses to the universal MHC class II–restricted epitopes are typically increased, the responses to the tumor antigen epitopes usually have been limited. We have hypothesized that increased immunogenicity to MHC class I–restricted epitopes may be achieved by immunizing with MHC class II–restricted epitopes derived from the same protein (12, 17).

In this study we evaluated whether active immunization with HER-2/neu helper peptide epitopes, each containing putative HLA-A2 MHC class I epitopes, would generate both CD4 and CD8 T-cell peptide and protein responses in vivo. In addition, we questioned whether HER-2/neu peptide–specific T cells, if they could be elicited, could recognize naturally processed and presented tumors. Finally, we questioned at what level immunity is elicited and how long immunity would last, as the ultimate goal is to generate long-term protective immunity against de novo formation or recurrence of tumor.

Methods

Clinical trial. Between August 1996 and August 1998, 19 patients with breast or ovarian cancer were enrolled in a phase I HER-2/neu peptide-based vaccine trial approved by the University of Washington's Human Subjects Division and the United States Food and Drug Administration. Eligibility was dependent upon subjects (a) being diagnosed with stage III/IV breast or ovarian cancer and having been treated for their primary and metastatic disease according to recommended disease-appropriate standards with surgery, chemotherapy, radiation therapy, or combined modality, (b) having a white blood cell count greater than 3.5 dl/ml, (c) showing HER-2/neu protein overexpression in the primary tumor or metastasis, (d) being off immunosuppressive drugs and chemotherapy for at least 30 days before enrolling, and (e) being HLA-A2 positive. Patients were tested for immune competence responsiveness to a minimum of two of seven recall antigens by skin testing with Multitest CMI (Pasteur Merieux Connaught Labs, Institut Merieux, Lyon, France). All patients signed a protocol-specific consent and received monthly vaccinations with three 15-amino acid (15-aa) HER-2/neu-derived peptides, p369–p384, p688–p703, and p971–p984, containing within each the putative HLA-A2-binding motifs p369–p377 (6), p689–p697 (7), and p971–p979 (18). Five hundred micrograms of each peptide (1.5 μg total peptide dose) were solubilized in 10 mM sodium acetate (pH 4.0) and admixed with 125 μg rhuGM-CSF (kindly supplied by Immunex Corp., Seattle, Washington, USA) as an adjuvant. The vaccine preparation was divided into two intradermal injections administered to the same draining lymph node site monthly for 6 months. Subjects underwent peripheral blood draws or a leukapheresis before and 30 days after each vaccination for immunologic monitoring.

Materials. The following peptides used in this study, either for immunization or in vitro use, were HLA-A2 flu matrix peptide (pFlu), GILGFVFTL (19); HLA-A2 cytomegalovirus (CMV) peptide, NLVPMVATV (20); and HER-2/neu peptides, p369-384, KIFGSLAFLPESFDGDPA (21), p688-703, RRLLQETELVEPLTPS (21), p971-984, ELVSEFSRMARDPQ (21), p369-377, KIFGSLAFL (6), p689-697, RLLQETELV (7), and p971-979, ELVSEFSRM (18). All peptides used for in vitro immunological monitoring were manufactured either by United Biochemical Inc. (Seattle, Washington, USA) or Multiple Peptide Systems Inc. (San Diego, California, USA), and all were greater than 95% pure as assessed by HPLC and mass-spectrometric analysis. Peptides used in vaccine preparations were manufactured by Multiple Peptide Systems (kindly provided by Corixa Corp., Seattle, Washington, USA) and approved for use in humans. Ficoll-Hypaque was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). RPMI-1640, HBSS, and PBS were purchased from Life Technologies (Rockville, Maryland, USA) and EHAA-120 from Biofluids (Rockville, Maryland, USA). [^{3}H] thymidine and [^{51}Cr] sodium chromate were purchased from NEN Life Science Products Inc. (Boston, Massachusetts, USA), human AB+ serum from Valley Biomedical Inc. (Winchester, Virginia, USA), sterile nitrocellulose-backed microfiltration 96-well plates from Millipore Corp. (Bedford, Massachusetts, USA), and streptavidin-alkaline phosphatase and AP-colorimetric reagents were from Bio-Rad Laboratories Inc. (Hercules, California, USA). Purified anti-IFN-γ (clone number 1-D1K) and biotin-conjugated anti-IFN-γ (clone number 7-B6-1) were purchased from Mabtech AB (Nacka, Sweden). Recombinant HER-2/neu protein domains (intracellular domain [ICD] and extracellular domain [ECD]) were provided by Corixa Corp. HLA testing was performed by the Puget Sound Blood Bank (Seattle, Washington, USA).

Cell lines. Epstein-Barr virus–transformed (EBV-transformed) lymphoblastoid cells (BLCLs) were produced from PBMCs using culture supernatant from the EBV-producing B95-8 cell line (American Type Culture Collection, Manassas, Virginia, USA). HLA-A2$^+$ BLCLs stably expressing human HER-2/neu were a kind gift from Steve Fling of Corixa Corp. The HER-2/neu–overexpressing cell lines SKOV3 and SKOV3-A2 and BLCLs were maintained in RPMI-1640 with L-glutamine, penicillin, streptomycin, 2-mercaptoethanol, and 10% FCS. The SKOV3-A2 tumor cells are the same as SKOV3 tumor cells, except they are stably transfected with a vector encoding HLA-A2 (22).

Preparation of PBMCs. PBMCs were obtained either by leukapheresis or 180–250 ml blood draws and isolated by density gradient centrifugation as described previously (21). Cells were analyzed immediately or aliquoted and cryopreserved in liquid nitrogen in freezing media (90% FBS and 10% dimethylsulfoxide) at a cell density of 25–50 × 10^6 cells/ml.

T-cell proliferation assays. HER-2/neu–specific T-cell proliferative responses were measured at base line, before each vaccination, and at the end of the study. T-cell proliferation was assessed using a modified limiting dilution assay designed for detecting low-frequency lymphocyte precursors based on Poisson distribution as described previously (21, 23). Data is reported as a stimulation index (SI), which is the mean of 24 experimental wells/mean of 24 no-antigen wells. An age-matched control population of 30 volunteer blood donors was analyzed similarly (data not shown). No volunteer donor had a response to HER-2/neu proteins or peptides. The mean and 3 SDs of the volunteer donor responses to all antigens (SI of 1.98) established a base line, therefore an SI greater than two was considered consistent with an immunized response.

Enzyme-linked immunosorbent spot assay. A 10-day enzyme-linked immunosorbent spot (ELIspot) assay was used to determine precursor frequencies of peptide-specific CD8 T lymphocytes as described previously (24). Briefly, on day 1, 2.5 × 10^5 PBMCs/well were plated into 96-well plates in six-well replicates in 200 μl of RPMI-1640 containing L-glutamine, penicillin,

streptomycin, and 10% AB serum (T-cell medium) in the presence or absence of 10 μg/ml peptide antigen or 0.5 U/ml tetanus toxoid. The cells were incubated at 37°C at 5% CO_2. On day 5, IL-2 was added to 10 U/ml. On day 8, 2.5×10^5/well irradiated autologous PBMCs and 10 μg/ml antigens were added. Also on day 8, nitrocellulose-backed 96-well plates (NC-plates) were coated with 10 μg/ml anti-IFN-γ Ab in PBS at 50 μl/well. On day 9 the NC-plate was washed three times with PBS and blocked for 2 hours with PBS containing 2% BSA, followed by three washes with PBS. On day 9, the cells were gently resuspended, pooled, centrifuged, and the media was replaced. The cells were transferred into the NC-plate in a volume of 100 μl/well in T-cell medium. The NC-plate was incubated at 37°C for a further 20–24 hours followed by washing three times using PBS containing 0.05% Tween-20. The plate was then incubated for 2.5 hours at room temperature in 50 μl/well PBS containing 5 μg/ml biotinylated anti-IFN-γ Ab, washed three times with PBS, and further incubated with 100 μl/well streptavidin-alkaline phosphatase at a dilution of 1:1,000 in PBS for 2 hours at room temperature. After washing three times in PBS, the plate was incubated with 100 μl/well AP-colorimetric substrate for 20–30 minutes, rinsed with cool tap water, and allowed to dry completely. Resultant spots were then enumerated using a dissecting microscope. Precursor frequencies were calculated by subtracting the mean number of spots obtained from the no-antigen control wells from the mean number obtained in the experimental wells. Statistical analysis was performed using the Student's *t* test (Microsoft Excel 97; Microsoft Inc., Bellevue, Washington, USA). Precursor frequencies to viral peptide antigens were also enumerated from peripheral blood from four $HLA\text{-}A2^+$ healthy, volunteer individuals for comparison purposes. Assay validation was established in preliminary studies using the HLA-A2, pFlu peptide over a PBMC range of $1.0\text{–}3.5 \times 10^5$ cells and also with the use of IFN-γ-coated polystyrene beads (24). These preliminary studies demonstrated that the assay is linear and precise between 2.0 and 3.5×10^5 PBMCs/well, has a detection limit of 1:100,000, and has a detection efficiency of 93%. The attributes of this assay, such as the limit of detection, are consistent with previously reported ELIspot methods (25, 26). The background number of spots per well, in the absence of antigen, was 10 ± 1 (mean ± SEM, $n = 180$). A positive response was defined as a precursor frequency that was both significantly ($P < 0.05$) greater than the mean of control no-antigen wells and detectable (i.e., >1:100,000). Although the ELIspot assay is sensitive and suitable for detecting low-level responses to vaccination (8, 13, 25), it is currently unknown if the calculated precursor frequencies represent actual numbers of antigen-specific cytolytic T cells in the peripheral blood.

Generation of antigen-specific T-cell lines and clones. Antigen-specific T-cell lines and clones were generated by culturing 25×10^6 PBMCs in T25 tissue-culture flasks in 20 ml of T-cell medium. For the generation of HER-2/neu-specific T-cell lines, PBMCs were cultured in 1 μM each of the HER-2/neu 9-aa peptides, p369–377, p689–697, and p971–979. For generation of p369–377-specific clones, p369–377 peptide was added to the flasks to 1 μm. The flasks were incubated at 37°C and 5% CO_2. On day 3 and every other subsequent day, IL-2 was added to 5 U/ml. On day 10, in vitro stimulation (IVS) was performed with peptide-pulsed, irradiated autologous PBMCs. The cultures were further incubated for an additional 10 days with periodic IL-2 administration. After the second IVS, the antigen-specific T-cell lines were examined for cytolytic activity as described below and in some cases were cloned. For cloning, bulk cultures were diluted to achieve approximately 0.3 viable cells/200 μl and plated onto flat-bottom 96-well plates in complete medium. Peptide-pulsed, irradiated autologous PBMCs (2.0×10^5) were added to each well in the presence of 50 U/ml IL-2. The wells were then tested for lytic activity in a [^{51}Cr]-release assay using 50 μl of cells from each well after 14 days. Positive wells were identified as those having specific activity of 5% or greater. The positive wells were transferred to new 96-well plates and subsequently restimulated with peptide-pulsed, irradiated autologous BLCLs. The cultures were eventually expanded and carried using IL-2 and peptide-pulsed, irradiated autologous BLCLs.

[^{51}Cr]-release assays. Cytolytic activity was measured using standard [^{51}Cr]-release assays. Effector cells were plated into 96-well plates at various effector-to-target (E/T) cell ratios. Targets used were either peptide- or protein-pulsed BLCL or the human HER-2/neu-overexpressing tumor cell lines, SKOV3 and SKOV3-A2. Targets were labeled with 200 μCi ^{51}Cr for 1–2 hours at 37°C. BLCLs were labeled simultaneously with 10 μM peptide. Before mixing with effectors, the targets were washed two times with medium and resuspended to 1,000 targets/100 μl. The reaction was carried out for 4 hours at 37°C, after which the plates were centrifuged and 50 μl of medium from each well was assayed for [^{51}Cr] content in a scintillation counter. The percentage of specific activity was calculated using the following equation: percentage of specific lysis = (sample well release – basal release) / (detergent release – basal release).

Results

Patients with advanced-stage HER-2/neu–overexpressing cancers can be safely immunized with peptide-based vaccines. Nineteen subjects were enrolled on trial (Table 1). The median age was 52 years (range 36–55), and the median time from last chemotherapy was 10 months (range 1–75). Fourteen subjects received six vaccinations, two received four vaccinations, two received three vaccinations, and one received one vaccination. Postvaccination data are presented on 18 subjects who received more than one vaccine. At enrollment, 18 subjects had positive-recall antigen testing. The one subject who was anergic received one vaccine. This subject withdrew

from the study because of worsening asthma. Toxicity was assessed using the National Cancer Institute Common Toxicity Criteria. Among all 19 subjects, there was one grade 2 skin rash characterized by a mild chronic urticaria that did not require treatment and resolved after completion of the vaccination series. Five subjects were followed a median of 12 months (range 7–17) after completing six vaccines.

Patients immunized with 15-aa HER-2/neu peptides develop HER-2/neu peptide– and protein–specific T-cell proliferative responses. T-cell proliferative responses were measured against 15-aa HER-2/neu peptides and the ECD and ICD proteins before, during, and after the vaccination series. As shown in Figure 1, before immunization proliferative responses were detected to p369–384 in 2 of 19 subjects (mean SI 1.0, range 0.6–2.4), to p688–703 in 2 of 19 subjects (mean SI 1.1, range 0.5–2.5), and to p971–984 in 1 of 19 subjects (mean SI 1.1, range 0.5–2.5).

After vaccination, 14 of 18 (83%) subjects had proliferative responses to at least one of the 15-aa HER-2/neu peptides contained within their vaccine formulations (Figure 2). After immunization, proliferative responses were detected to p369–384 in 14 of 18 subjects (mean SI 6.4, range 1.0–35.6), to p688–p703 in 7 of 18 subjects (mean SI 2.4, range 1.0–6.4), and to p971–984 in 10 of 19 subjects (mean SI 4.2, range 1.0–26.1) (Figure 1). The differences in means between the preimmunization responses and the maximal postimmunization responses were significant for all the peptides (p369–384, $P = 0.003$; p688–703, $P = 0.001$; p971–984, $P = 0.02$). Overall, new immunity was generated to p369–384, p688–703, and p971–984 helper peptides in 67%, 33%, and 50% of subjects, respectively (Figure 2). As a comparison, the mean maximal response to tetanus toxoid in the patient population was an SI of 14.8 (range 1.0–76.8) (Figure 1).

Table 1
Patient demographics

Diagnosis	Number of patients
Breast cancer	19
Stage III	4
Stage IV	14
Ovarian cancer	1
Stage III	1
Age, median years (range)	52 (36–55)
Time from last chemotherapy, median months (range)	10 (1–75)

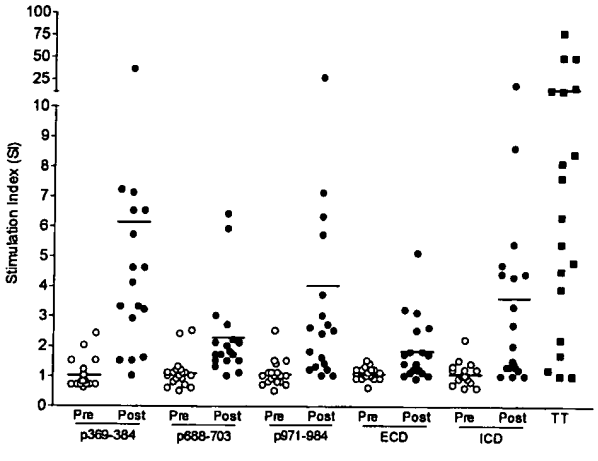
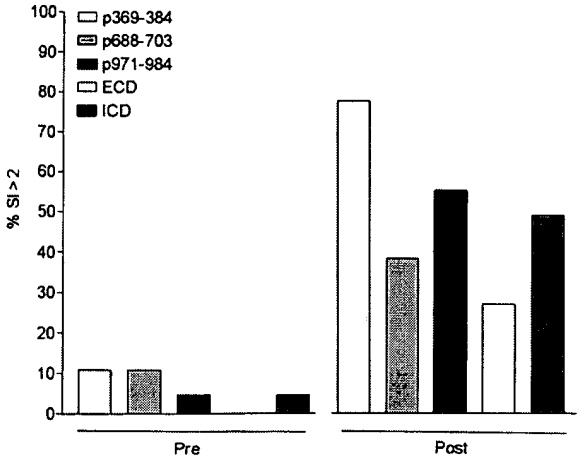


Figure 1
Patients immunized with a 15-aa HER-2/neu peptide–based vaccine develop HER-2/neu peptide–specific and protein–specific T-cell proliferation responses. Shown are the preimmunization (open circles) and maximal postimmunization (filled circles) proliferative responses (SI) for the HER-2/neu peptides, p369–384, p688–703, p971–984, and the HER-2/neu protein domains, ECD and ICD. For comparison, the maximal responses to tetanus toxoid (TT) are shown. Each symbol represents a measurement from a single unique subject, calculated from 24 replicates. The solid lines indicate the mean SI for the group.

Patients immunized with peptides also developed responses to the naturally processed and presented HER-2/neu protein. As shown in Figure 1, before immunization proliferative responses were detected to the ECD in 0 of 19 subjects (mean SI 1.1, range 0.6–1.5) and to the ICD in 1 of 19 (mean SI 1.1, range 0.6–2.2). After immunization, proliferative responses were detected to ECD in 5 of 18 subjects (mean SI 1.9, range 1.0–5.1) and to ICD in 9 of 18 subjects (mean SI 3.9, range 1–18). The differences in the mean postimmunization responses were significantly higher than the mean preimmunization SIs for both proteins (ECD, $P = 0.001$; ICD, $P = 0.004$). Overall, new immunity was generated to ECD protein in 28% and to ICD protein in 50% of subjects.

Patients immunized with 15-aa HER-2/neu peptides increase T-cell precursors to the HLA-A2 9-aa peptide epitopes contained within the longer peptide sequences of the vaccine peptides. Generation of HER-2/neu T-cell precursors to the 9-aa peptides, p369–377, p689–697, and p971–979, were evaluated in patients using an IFN-γ–based ELIspot (Figure 3). Figure 3a demonstrates that study patients had similar levels of viral-specific T-cell precursors compared with the levels of viral-specific precursors detected in a cohort of HLA-A2 volunteers. Before vaccination, IFN-γ–producing CD8 T-cell responses, defined as HER-2/neu peptide-specific precursors/10^6 PBMCs, were detectable to p369–377 in 2 of 15 (mean 12, range 0135), to p689–697 in 0/15 (mean 0, range 0-0) and to p971–979 in 3/15 (mean 21, range 0–217) subjects. As shown in Figure 3, after immunization CD8 T-cell responses were detected to p369–377 in 10 of 15 subjects (mean 75, range 0–471), to p688–703 in 5 of 15 subjects (mean 20, range 0–143), and to p971–984 in 12 of 15 subjects (mean 63, range 0–185). Overall, new CD8 T-cell immunity was generated to p369–377 in 62%, to p689–697 in 31%, and to p971–979 in 54% of subjects (Figure 4). The pre- and postimmunization responses to tetanus toxoid and the HLA-A2 peptides from influenza virus and CMV were not significantly changed ($P > 0.05$) as a result of vaccination with HER-2/neu peptides (data not shown).

Figure 2
The majority of patients could be immunized to HER-2/neu. Data are shown as the percentage of the population before immunization and after immunization that had a positive proliferation response (SI > 2) to each of the peptides in the vaccine, p369-384 (light gray bar), p688-703 (gray bar), p971-984 (filled bar), as well as to the HER-2 protein domains, ECD (open bar) and ICD (dark gray bar). The mean (range) preimmunization SI of patients considered as having a positive response to p369-384 was 2.4 (one patient only), to p688-703 was 2.5 (2.4-2.5), to p971-984 was 2.5 (one patient only), to ECD (no patients), and to ICD was 2.2 (one patient only). The mean (range) postimmunization SI of patients considered as having a positive response to p369-384 was 7.5 (2.9-35.6), to p688-703 was 3.5 (2.1-6.4), to p971-984 was 6.2 (2.4-26.1), to ECD was 3.3 (2.5-5.1), and to ICD was 6.2 (2.7-18).



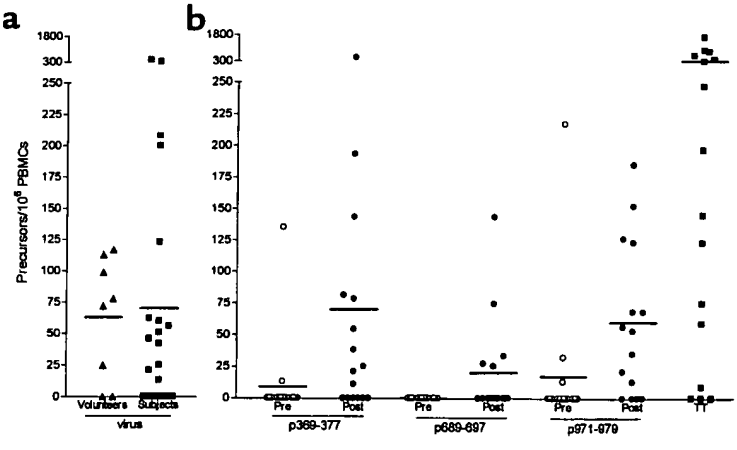
Peptide-specific T cells can lyse HLA-matched cells expressing HER-2/neu protein. Previous studies of peptide immunization indicate peptide-specific T cells may not have the capacity to lyse tumors (9, 27, 28). Thus, we assessed whether HER-2/neu peptide-specific T cells could lyse HLA-A2, HER-2/neu-expressing cell lines. As an example, Figure 5 demonstrates cytolytic activity against an HLA-A2 BLCLs transfected with HER-2/neu in a breast cancer patient after immunization. Postimmunization precursor frequencies in this representative subject after vaccination were 81, 27, and 56 precursors/10^6 PBMCs to p369-377, p689-697, and p971-979, respectively. The patient had no pre-existing peptide-specific T-cell precursors before immunization. A representative T-cell line established on this patient demonstrated a 40:1 E/T ratio, 22% lysis to p369-377, 35% lysis to p689-697, and 37% lysis to p971-979 (Figure 5). Furthermore, peptide-specific T cells were able to lyse HLA-A2$^+$ BLCLs expressing HER-2/neu protein (25% at 40:1 E/T). In an additional example, 21 CD8 peptide-specific T-cell clones were generated from an ovarian cancer patient, 0756, after vaccination (29). Shown in Figure 6 is the cytolytic activity of a representative peptide-specific clone against p369-377-loaded HLA-A2 BLCLs (25% at 40:1 E/T) or tumor cells expressing both HER-2/neu and HLA-A2 (18% at 40:1).

Patients immunized with 15-aa HER-2/neu peptides maintain HER-2/neu immune responses after immunizations have ended. To determine if CD8 T-cell responses were maintained after active immunization, five patients were followed between 7 and 17 months after the end of vaccination. All five of the patients maintained responses to two or more the HLA-A2 9-aa epitopes contained within their vaccine peptides (Table 2). At a median of 12 months after the last vaccination, the mean (n = 5 patients) precursor frequency, expressed as peptide-specific precursors/10^6 PBMCs, to p369-377 was 68 (range 38-118), to p689-697 was 22 (range 0-74), and to p971-979 was 43 (range 0-68).

Discussion

The cytolytic T-cell response is believed to be the critical immune effector arm in mediating potential antitumor immunity. Thus, studies of tumor immunity primarily have focused on the identification of MHC class I epitopes for tumor-associated antigens such as MAGE-1 (30), NY-ESO-1 (31), HER-2/neu (6, 7), tyrosinase (32), and gp-100 (33). In in vitro experiments, these epitopes, when presented in the context of MHC class I, activate CD8 T cells that can directly lyse tumors. Therefore, several clinical trials have been conducted assessing the feasibility and efficacy of cancer vaccination with peptide-

Figure 3
Patients immunized with a 15-aa HER-2/neu vaccine increase T-cell precursors to the encompassed HLA-A2 9-aa peptides. (**a**) Combined ELIspot responses (precursors/10^6 PBMCs) to HLA-A2-binding epitopes of influenza and CMV virus are shown. Data are from normal HLA-A2 volunteers (triangles) and study subjects (squares), with the mean delineated by a bar. (**b**) Preimmunization and maximal postimmunization ELIspot responses (precursors/10^6 PBMCs) to the HLA-A2 HER-2/neu peptides, p369-377, p689-697, p971-979, in subjects are shown. For comparison, the maximal responses to TT are shown. Each symbol represents a measurement from a single unique subject, calculated on six replicates.



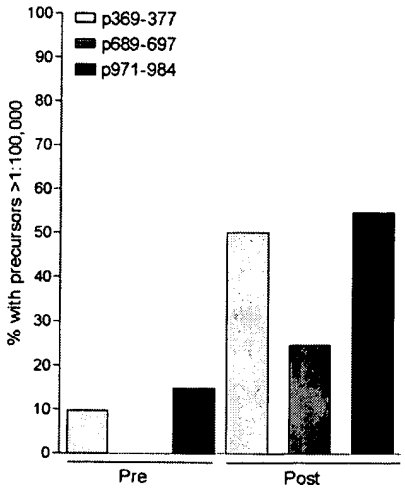


Figure 4
The majority of patients could be immunized to HLA-A2 HER-2/neu peptides. Data are shown as the percentage of the population before immunization and after immunization that had a detectable ELIspot response to each of the HER-2/neu, HLA-A2 peptides, p369-377 (light gray bar), p689-697 (dark gray bar), p971-979 (filled bar). The mean (range) preimmunization HER-2/neu-specific precursor frequency of patients considered as having a positive response to p369-377 was 125 (32-217, two patients only), to p689-697 (no patients), and to p971-979 was 74 (13-135, two patients only). The mean (range) maximal postimmunization HER-2/neu-specific precursor frequency of patients considered as having a positive response to p369-377 was 111 (11-417), to p689-697 was 60 (25-143), and to p971-979 was 97 (13-185).

based MHC class I peptides to generate tumor-specific CD8 T cells. Several problems have been identified with MHC class I peptide vaccination, including the inability to generate (a) peptide-specific precursors that directly recognize naturally processed antigen, (b) a significant precursor frequency, and (c) long-lasting immunity.

The requirement of CD4 help to initiate and sustain a CD8 response is well established and has led to the development of antitumor vaccines that attempt to induce both T-cell subsets (1, 34). In the absence of defined tumor-antigen MHC class II epitopes needed to activate CD4 T cells, immunization strategies have been employed combining tumor antigen MHC class I epitopes with universally recognized MHC class II epitopes such as PADRE (35) and the promiscuous epitopes of tetanus toxoid (36). Although immunity to the helper epitopes is usually robust, responses to the antigen of interest have been limited. For example, Brander and colleagues reported that inclusion of the promiscuous tetanus epitope, p30, into a vaccine formulation containing an HIV HLA-A2 peptide epitope did not result in immunization to the HIV epitope but significantly reactivated the memory response to the tetanus peptide (37). Similarly, PADRE was unable to significantly induce immunity to two human papilloma virus-derived, HLA-A2 peptides in a phase I clinical trial (16). In contrast to these strategies, providing CD4 help within the same antigenic background has been successfully used to boost CD8 responses (12, 17). In fact, immunizing with an MHC class II epitope that encompasses an MHC class I-binding motif within its natural sequence resulted in effective immunity in a murine lymphocyte choriomeningitis virus model (17). Previous investigations by our group identified putative T-helper epitopes of the HER-2/neu protein that contained HLA-A2 motifs (2). By providing HER-2/neu-specific MHC class II and MHC class I epitopes simultaneously, we hoped to overcome the problems associated by immunizing with MHC class I epitopes alone.

The peptide-specific T cells that were generated in vivo in the present study were able to lyse tumor cells. The inability of peptide-specific T cells generated by vaccination to directly recognize naturally processed and presented antigen has been reported for some MHC class I epitopes, including those derived from HER-2/neu (9)

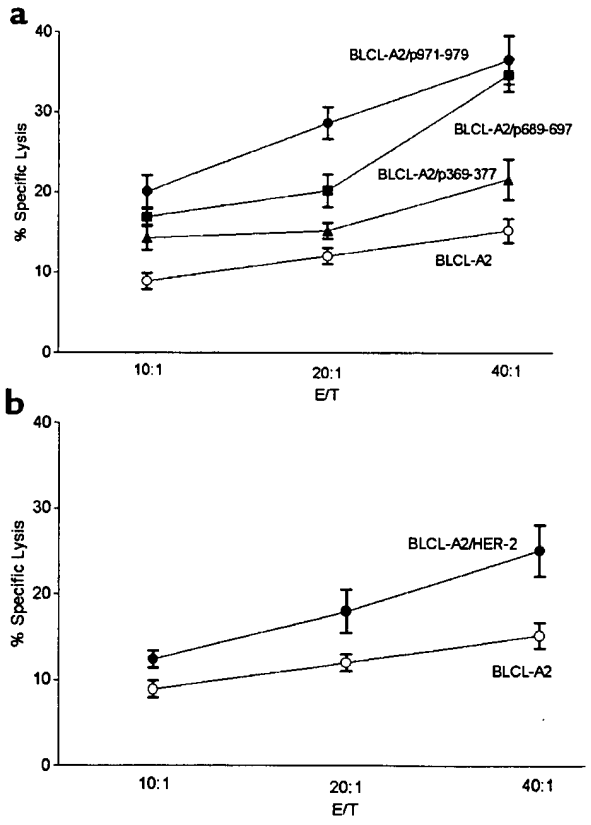


Figure 5
Peptide-specific T cells isolated from a breast cancer patient after immunization can lyse HLA-A2 cells overexpressing HER-2/neu protein. PBMCs from a representative patient, 0107, were examined for cytolytic activity against BLCL-A2 alone (open circles), peptide-loaded A2-BLCLs (filled symbols), or HER-2/neu-expressing BLCL-A2 (open squares) at three different E/T ratios. The peptides (p369-377, p689-697, p971-979) used to pulse the BLCL-A2 were the HLA-A2-binding peptides encompassed in the 15-aa HER-2/neu vaccine. Each point represents the mean of three replicates.

Table 2
CD8 T-cell responses are maintained after vaccination

Subject	p369–377		p689–697		p971–979	
	Maximal response	Latest response[A]	Maximal response	Latest response[A]	Maximal response	Latest response[A]
8302	1:12,800	1:21,300	1:30,300	1:91,000	1:25,000	1:25,000
4716	1:8,500	1:8,500	<1:100,000	<1:100,000	1:18,700	1:20,000
4723	1:5,200	1:18,500	1:7,000	<1:100,000	1:14,700	1:14,700
2859	1:26,300	1:26,300	1:13,500	1:13,500	1:76,900	<1:100,000
0107	1:12,300	1:12,300	1:37,000	1:37,000	1:17,900	1:17,900

Data are presented as ratio of antigen-specific T cells to PBMCs. [A]Response measured between 7 and 17 months (median = 12) after final vaccination.

and MART-1 (28). Peptide-specific T cells that do not respond to endogenous antigen may be detected after immunization if target cells (e.g., tumor cells) do not naturally process or present the epitope in sufficient quantities to stimulate recognition. Alternatively, immunization with excessive quantities of class I–restricted peptide may result in the generation of peptide-specific T cells that are of low affinity and would not be activated by the level of naturally occurring peptide in tumor cells. Vaccinating with longer peptides encompassing class I motifs, as in the present study, may allow processing of the peptide and presentation in class I MHC at levels that more closely mimic those present on tumors.

The majority of patients in the current study generated viral-like levels of HER-2/neu–specific CD8 T-cell precursors. Immunization with MHC class I cancer vaccines often has resulted in undetectable or low-level immune responses. For example, Pass and colleagues reported that only two of seven (29%) melanoma patients vaccinated with the gp100 HLA-A2–binding motif, g208, developed detectable peptide-specific precursors (8). In a parallel study, they also observed no detectable response in another cohort vaccinated against the HLA-A2 MART-1_{27-35} epitope (8). Our success at generating high levels of peptide-specific precursors to HER-2/neu HLA-A2 peptides was most likely due to the patient population selected, having either low-level or nondetectable disease and an excellent performance status. Scheibenbogen and colleagues have demonstrated that the presence of antigen-specific immune reactivity in melanoma patients can be correlated with disease being in remission (38). Our goal, like that in infectious disease, is to develop vaccination strategies that prevent disease rather then treat disease. The inability of vaccines to eradicate actively growing tumors has been clearly shown in animal models (39). In our study 86% of patients generated increased frequencies of HER-2/neu-specific CD8 T cells. The mean HER-2/neu (all peptides) precursor frequency of 49/10^6 PBMCs was very similar and within one SD to the mean viral (flu and CMV) precursor frequency of 65/10^6 PBMCs. Furthermore, the levels of viral precursors measured in the present study are consistent with those observed by Scheibenbogen in a cohort of melanoma and noncancer-bearing patients (38). These findings together raise the important question as to whether levels of cancer immunity that are similar to viral immunity would be sufficient to protect against cancer relapse. This question can be answered only in the context of clinical studies designed to correlate level of immunity generated after immunization with protection from cancer relapse or development of disease.

Our vaccination strategy also resulted in persistent peptide-specific T-cell precursors. Over the past decade, immunization with CD8 T cell–inducing epitopes was associated with only short-lived responses (40). However, strategies are being developed to lengthen the duration of the response. It is likely that our strategy resulted in long-lived CD8 T-cell responses due to the concurrent activation of CD4 T cells. Both murine antiviral and antitumor models have clearly established the important role of CD4 T cells in maintaining a persistent CD8 T cell response (14, 34, 41). Using a different strategy, Stewart and Rosenberg have found that gp100-specific CD8 T-cell responses are long-lived in melanoma patients immunized with a modified gp100 MHC class I–binding peptide (42).

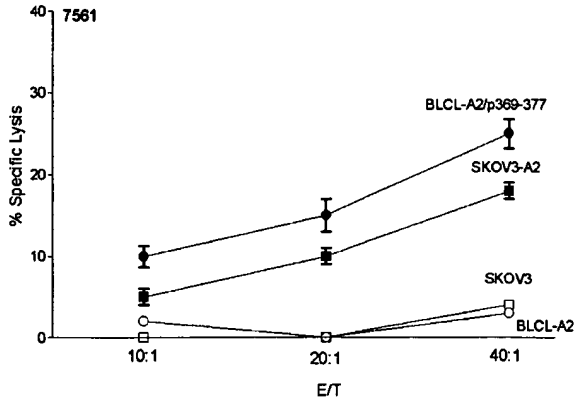


Figure 6
Peptide-specific T-cell clones isolated from an ovarian cancer patient after immunization can lyse HLA-A2$^+$ tumor cells overexpressing HER-2/neu protein. A p369–377–specific clone was examined for cytolytic activity against BLCL-A2 alone (open circles), p369–377–loaded BLCL-A2 (filled circles), or the HER-2/neu–overexpressing tumor cells, SKOV3 (open squares) and SKOV3-A2 (filled squares). SKOV3-A2 are SKOV3 cells stably expressing HLA-A2. Each point represents the mean of three replicates (± SEM). The absence of errors indicates a standard of the mean less than 1%.

In summary, the primary use of cancer vaccines is most likely to prevent, rather than eradicate, malignancy. In this study immunization of cancer patients against HER-2/neu was demonstrated with a peptide-based vaccine consisting of helper T-cell epitopes, each containing an HLA-A2 motif. Active immunization resulted in the generation of both CD4 and CD8 T-cell immunity. The resulting peptide-specific T-cell precursors recognized naturally processed HER-2/neu protein and the immunity was long-lived.

Acknowledgments

We would like to express our gratitude to the patients who participated in this study and to the oncologists who referred their patients to us. This work is supported for K.L. Knutson by a fellowship from the Department of Defense Breast Cancer Program and for M.L. Disis by grants from the NIH, the National Cancer Institute (R01 CA75163), and the Department of Defense Breast Cancer Program. Patient care was conducted through the Clinical Research Center Facility at the University of Washington, which is supported through the NIH grant MO1-RR-00037. We are grateful for the expert nursing care and protocol coordination by Lynne Fitzsimmons and Donna Davis.

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Commentary

Progress in cancer vaccines by enhanced self-presentation

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Early efforts toward human cancer immunotherapy were hindered by the absence of identified antigens to target by vaccination or cell therapy. In the past decade, studies examining $CD8^+$ and $CD4^+$ T cell responses to melanoma and other tumors have uncovered several classes of proteins that give rise to peptides presented by MHC molecules. These include (*i*) differentiation antigens expressed in the tumor and its normal tissue counterpart e.g., tyrosinase; (*ii*) cancer-testes antigens expressed in the testes and a variety of malignancies e.g., MAGE-1; and (*iii*) tumor-specific antigens that arise from mutations in tumor cells e.g., CDK-4 (1–4). The observation that autoimmune depigmentation of the skin (vitiligo) developed in some melanoma patients with tumor regression after IL-2 therapy provided optimism that antigens derived from self-proteins might have utility as targets for immunotherapy (5). Animal model studies demonstrated that T cell immunity could be elicited to tissue-specific self-proteins and promote tumor regression without severe autoimmune injury and provided rationale for clinical vaccine trials targeting self-proteins (6–9). However, with a few exceptions the results of vaccination for human malignancy have been disappointing (10–13). It appears the problem is caused by central and peripheral tolerance mechanisms that limit the repertoire of self-reactive T cells to those of low avidity to prevent autoimmunity, making it difficult to elicit a T cell response sufficient to eradicate tumor (14–16). Recent studies have suggested that the balance between immunity and tolerance is regulated at the immunologic synapse between T cells and specialized bone marrow-derived dendritic cells (DC) that present antigens to T cells (17–19). In a recent issue of PNAS, Fong *et al.* (20) provide evidence that a key to inducing tumor immunity may lie at this interface. The investigators target carcinoembryonic antigen (CEA), a glycoprotein expressed in normal gastrointestinal (GI) and genitourinary epithelial cells and in most adenocarcinomas of GI origin, 50% of breast cancers, and 70% of non-small-cell lung cancer (21). They demonstrate that vaccination of patients with DC displaying a CEA peptide altered to promote more efficient engagement of T cell receptors elicited high levels of $CD8^+$ cytotoxic T cells (CTL) specific for native CEA. Remarkably, the induction of CTL was associated with tumor regression in some patients with advanced cancer without autoimmunity (20).

The balance between immunity and tolerance is regulated at the immunologic synapse between T cells and specialized bone marrow-derived dendritic cells that present antigens to T cells.

The activation and differentiation of tumor-reactive T cells by vaccination has centered on manipulating two variables, the antigen-presenting cell (APC) and antigen. DC are specialized APCs strategically located in tissues where in their immature form they are proficient at capturing and processing antigen (22). DC maturation is induced by pathogens or inflammatory mediators and is characterized by expression of CCR-7, which promotes trafficking to T cell zones of secondary lymphoid organs, and up-regulation of MHC, costimulatory, and adhesion molecules, which collectively permit the formation of a synapse with naïve T cells expressing a T cell receptor of sufficient avidity (22–24). In animal models, inoculation of DC pulsed with peptides, transfected with RNA- or DNA-encoding tumor antigens, or fused to tumor cells induces tumor-specific immunity (25). Thus, DC have been viewed as the vaccine vehicle of choice for overcoming tolerance to self-antigens.

An obstacle for human studies is obtaining sufficient cells for vaccination because DC comprise <1% of leukocytes in the blood (22). One approach is to administer Flt-3 ligand (Flt3-L), which activates the Flt-3 receptor tyrosine kinase and serves as a growth and differentiation factor for hematopoietic progenitors and expands DC *in vivo* (26). In normal mice, administration of Flt-3L increased both myeloid and lymphoid DC subsets in blood, lymph nodes, and spleen (27). In mice bearing an immunogenic MCA sarcoma, Flt-3L caused infiltration of DC into the tumor and tumor regression mediated by $CD8^+$ CTL (28). Administration of Flt-3L to normal individuals and patients with cancer also increased DC numbers in the blood and infiltration of DC into tumor metastases but did not cause tumor regression (29, 30). Fong *et al.* (20) show that administration of Flt-3L can facilitate procurement of DC to permit evaluation of larger cell doses in vaccine studies. The yield of DC obtained by leukapheresis was increased by >60-fold after Flt-3L administration, and with brief *in vitro* culture the mobilized DC up-regulated expression of CD80, CD83, CD86, MHC, and CCR-7 molecules consistent with acquisition of a mature phenotype (20).

Flt-3L assists in obtaining APCs for vaccination but the formidable task is to display tumor-associated self-antigen in a form that is effective for inducing T cell responses. Insight into how this might be accomplished can be derived from the observation that the quality and duration of T cell receptor signaling at the synapse between DC and T cell influences T cell activation (31). It follows that the ability of a peptide antigen to elicit responses will be related to its affinity for the MHC molecule, determined by the presence of favored amino acids at critical anchor positions involved in MHC binding and by the affinity of the MHC-peptide complex for the T cell receptor. Thus, altered peptide ligands containing amino acid substitutions at residues that anchor the peptide in the MHC binding groove or contact the T

See companion article on page 8809 in issue 15 of volume 98.

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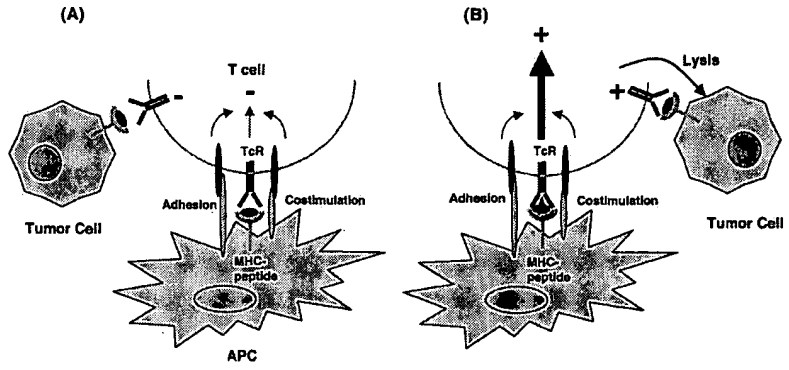

Fig. 1. Altering the peptide ligand at the immunologic synapse elicits an effector T cell response to a self-antigen. (*A*) Self-antigens are displayed as peptides bound to MHC on professional APC but because of the low avidity of the T cell repertoire, insufficient signaling is generated to induce an effector T cell response and tumor cells expressing the self-antigen are ignored. (*B*) Altering amino acid residues of the peptide that protrude out from the MHC molecule and contact the T cell receptor (TcR) improves the affinity of the interaction and promotes signaling and T cell activation. The result is expansion of self-reactive T cells and differentiation to effector cells that have a lower threshold for activation and recognize tumor cells expressing the native self-peptide.

cell receptor can inhibit or enhance T cell signaling (Fig. 1) (31–34). The relevance for vaccination was demonstrated in murine tumor models and patients with melanoma in which peptides modified at MHC anchor residues were shown to elicit superior T cell responses to the original unmodified antigen (13, 35). Similarly, vaccination with an altered peptide for a class I-restricted murine tumor antigen that increased the stability of the interaction between MHC-peptide complex and the T cell receptor also enhanced T cell responses and tumor protection when compared with the natural epitope (36).

In the study by Fong *et al.* (20), this principle that peptides modified in T cell receptor contact residues can enhance immunogenicity and break tolerance to a self-protein is extended to a candidate human tumor antigen, CEA. Previous trials using recombinant viruses encoding CEA to vaccinate patients elicited a low frequency of CTL specific for a nonamer CEA peptide (CAP1) presented by HLA A2 (37). Subsequently, DC pulsed with CAP1 was used as a vaccine but failed to induce clinical responses in patients with CEA-positive malignancy (38). CAP1 contains preferred amino acids at anchor positions and binds HLA A2 with high affinity, suggesting alterations to promote MHC binding would not improve immunogenicity. However, an altered CAP1 ligand (CAP1-6D) containing aspartic acid in place of asparagine at position 6, which is predicted by crystallographic data to protrude toward the T cell receptor, was more effective than CAP1 for eliciting tumor-reactive $CD8^+$ CTL *in vitro* and stimulated greater phosphorylation of the T cell receptor ζ chain and ZAP-70 (39). Fong *et al.* (20) now show that vaccination of cancer patients with DC pulsed with CAP1-6D and a keyhole limpet hemocyanin antigen to provide T cell help induced $CD8^+$ CTL that lyse CEA-expressing tumor cells. In five patients, the magnitude of CTL responses achieved with vaccination was substantial, exceeding 1% of $CD8^+$ T cells in the blood as measured by staining with a CAP1 or CAP1-6D tetramer. These tetramer-positive cells exhibited a $CD45RA^+$, $CD44^+$, $CD27^-$, and $CCR7^-$ phenotype consistent with differentiation to effector T cells.

The striking and hopeful finding in the study by Fong *et al.* (20) was the regression of metastatic colon cancer in two of the 12 vaccinated patients. One additional patient had a mixed response and two others had stable disease. These clinical responses correlated with increases in tetramer-positive T cells, implicating $CD8^+$ CTL in tumor regression. Colon cancer has not been considered responsive to immunotherapy, and these results are dramatic in view of the advanced stage of the tumors. To build on these results it will be important to discern the contribution to antitumor activity provided by each of the components of the regimen, which included Flt-3L, DC, altered peptide ligand, and the keyhole limpet hemocyanin helper antigen. Obstacles to tumor eradication identified in prior studies may emerge, including outgrowth of antigen loss variants (40), failure of T cells to infiltrate tumor masses (41), anergy or deletion of reactive T cells (42, 43), and autoimmunity if the self-reactive T cell response elicited by vaccination is too vigorous (44, 45). Nevertheless, these provocative findings provide optimism that other interventions at the DC:T cell interface such as augmenting costimulation or reducing inhibitory signals may have utility for human cancer vaccines (46, 47).

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ecember 12, 1997 VOLUME 272 NUMBER 50

0021-9258
HA3 272(50) 31209–31936 (1997)

THE Journal of Biological Chemistry

Published by the American Society for Biochemistry and Molecular Biology

FOUNDED BY CHRISTIAN A HERTER

AND SUSTAINED IN PART BY THE CHRISTIAN A HERTER MEMORIAL FUND

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The Journal of Biological Chemistry (ISSN 0021-9258) is published weekly by the American Society for Biochemistry and Molecular Biology, Inc., 9650 Rockville Pike, Bethesda, MD 20814. Volume 273 for 1998: Institutions: United States, $1,500; Foreign, $1,750. Single copies of the Journal are $30. (Note: Maryland and Canada add applicable taxes, unless a tax-exempt certificate number is shown.) The GST number for Canadian subscribers is 13037 0018 RT. C.P.C Int'l Pub Mail # 0060119. Periodicals postage paid at Bethesda, MD 20814, U.S.A. and at additional mailing offices. Printed in the U.S.A. Subscription rates for the on-line JBC are available at http://www.jbc.org. POSTMASTER: Send address changes to THE JOURNAL OF BIOLOGICAL CHEMISTRY at P.O. Box 830399, Birmingham, AL 35283-0399, U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 50, Issue of December 12, pp. 31636–31640, 1997
Printed in U.S.A.

hsp110 Protects Heat-denatured Proteins and Confers Cellular Thermoresistance*

(Received for publication, September 3, 1997, and in revised form, September 12, 1997)

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The 110-kDa heat shock protein (hsp110) has long been recognized as one of the primary heat shock proteins in mammalian cells. It belongs to a recently described protein family that is a significantly diverged subgroup of the hsp70 family and has been found in organisms as diverse as yeast and mammals. We describe here the first analysis of the ability of hsp110 to protect cellular and molecular targets from heat damage. It was observed that the overexpression *in vivo* of hsp110 conferred substantial heat resistance to both Rat-1 and HeLa cells. *In vitro* heat denaturation and refolding assays demonstrate that hsp110 is highly efficient in selectively recognizing denatured proteins and maintaining them in a soluble, folding-competent state and is significantly more efficient in performing this function than is hsc70. hsp110-bound proteins can then be refolded by the addition of rabbit reticulocyte lysate or hsc70 and Hdj-1, whereas Hdj-1 does not itself function as a co-chaperone in folding with hsp110. hsp110 is one of the principal molecular chaperones of mammalian cells and represents a newly identified component of the primary protection/repair pathway for denatured proteins and thermotolerance expression *in vivo*.

It has been long recognized that the major heat shock proteins (hsps)[1] of mammalian cells are observed at 28, 70, 90, and 110 kDa (1–3) and other hsp families, *e.g.* hsp60 and hsp40, have been subsequently identified. All of these stress protein groups have been intensively studied, excluding the hsp110 species. The cloning of hsp110 from hamster, mouse, yeast, arabadopsis, and a variety of other species has been recently described (4–11, 29, 30). Moreover, as is the case with the hsp70 family, multiple members of the hsp110 family have also been found in individual organisms (8–11). These studies indicate that hsp110 is a significantly enlarged and diverged relative of the hsp70 family of proteins but also includes unique sequence components. The notable constitutive expression and stress inducibility of hsp110 is highly suggestive of a major role in unstressed cells as well as in the heat shock response and the expression of thermotolerance (1, 2). A description of the heat shock response in eucaryotes is not possible without an understanding of the roles played by this major stress protein. We describe here an analysis of the characteristics of hsp110, both *in vivo* and *in vitro*.

* This work was supported in part by Public Health Service Grant GM45994. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: hsp, heat shock protein; RRL, rabbit reticulocyte lysate; hsc, constitutively expressed hsp70; SSE, yeast stress seventy E family.

EXPERIMENTAL PROCEDURES

Purification of Recombinant His-tagged HSP110—cDNA for hsp110 was cloned into pRSET vector (Invitrogen), resulting in introduction of a His_6-(enterokinase recognition sequence)-Arg-Ser tag to the amino terminus of hsp110 (pRSET-hsp110). pRSET-hsp110 was transformed into *Escherichia coli* strain JM109(DE3) cells. The transformant containing pRSET-hsp110 was grown at 37 °C in LB medium with ampicillin until the OD reached 0.6, when the expression of His-hsp110 was induced by the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside during further incubation at 30 °C for 5 h. Cells were lysed in 20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole, 0.1% Nonidet P-40, using lysozyme treatment and sonication. The lysate was centrifuged at 25,000 × *g* for 30 min, and His-hsp110 was purified from the supernatant on Ni^{2+}-nitrilotriacetic acid-agarose columns (QIAGEN, Inc.) following manufacturer's instruction. Briefly, the supernatant was loaded on the column, washed with sonication buffer, washed with wash buffer containing 20 mM Tris-HCl, pH 7.8, 0.5 M NaCl, 60 mM imidazole, 10% glycerol, and hsp110 was eluted with wash buffer containing 500 mM imidazole (instead of 60 mM). The eluent was dialyzed against 20 mM Tris-HCl, pH 7.8, 150 mM NaCl for 48 h and concentrated by sprinkling polyethylene glycol 8000 or by ultrafiltration using Centricon 50. The concentration of proteins was determined using the Bio-Rad protein assay kit.

hsp110 Expression in Tissue Culture Cells—Myc epitope (EEQKLISEEDLLR) was added to the COOH terminus of hsp110 using polymerase chain reaction amplification of the fragment from *Sst*I site to the stop codon of hsp110 cDNA. Amplification was performed using primers containing the *Sst*I site, the inserted Myc epitope, and the stop codon. The nucleotide sequence of the amplified region was verified by DNA sequencing. The Myc-tagged cDNA for hsp110 was cloned into pUHD10-3 (gift from Dr. H. Bujard), which contains the tetracycline-dependent promoter (tet-hsp110). The plasmid tet-hsp110 was cotransfected with the thymidine kinase-hygromycin plasmid into Rat-1-R12 (ATCC) and tetracycline-dependent transactivator 1 (HtTA-1) (ECACC) cells, which are Rat-1 and HeLa cell lines, respectively, transformed with the tetracycline-dependent transactivator. Doxycycline (tetracycline derivative, 2 ng/ml) was added to the culture medium (Dulbecco's modified Eagle's medium + 10% fetal bovine serum + penicillin-streptomycin-neomycin antibiotic mixture). For stable clone selection, 150 μg/ml hygromycin and 400 μg/ml G418 were included in the culture medium. hsp110-Myc-expressing clones were screened by Western analysis with monoclonal antibody against Myc epitope in the absence of doxycycline. Selected clones were routinely maintained in medium containing doxycycline, hygromycin, and G418.

Thermal Aggregation Experiments—150 nM luciferase (Boehringer Mannheim) or 75 nM citrate synthase (Sigma) alone or with bovine serum albumin, ovalbumin, hsp110, or hsc70 (StressGen, Inc.) were equilibrated to room temperature in 25 mM Hepes, pH 7.9, 5 mM magnesium acetate, 50 mM KCl, 5 mM β-mercaptoethanol, and 1 mM ATP (as indicated) followed by incubation at 43 °C in a thermostated cuvette. Light scattering by protein aggregation was determined by measuring the increase of optical density at 320 nm with a spectrophotometer. After measuring the light scattering, the samples were transferred to microcentrifuge tubes and centrifuged for 15 min at 16,000 × *g* at 4 °C, and the supernatant and pellet were separated. The samples taken before centrifugation were considered as total protein. Total, supernatant, and pellet fractions were run on SDS-polyacrylamide gel electrophoresis and probed with anti-luciferase (or anti-hsp110) antibody.

Detection of the Interaction between Luciferase and hsp110 by Immunoprecipitation—Luciferase (150 nM) was incubated with hsp110 (150 nM) in buffer used for aggregation experiments at room temperature or 43 °C for 30 min and chilled on ice. Anti-hsp110 antibody was added to

the luciferase solution, and the buffer was adjusted to radioimmune precipitation buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 1 mg/ml ovalbumin and incubated for 1 h at 4 °C followed by incubation with protein A-Sepharose for 18 h at 4 °C. The protein A-Sepharose pellet was collected and washed six times, and the pellet was resolved in SDS-polyacrylamide gel electrophoresis and subjected to Western analysis with anti-hsp110 or anti-luciferase antibody.

Luciferase Reactivation Experiments—Luciferase (150 nM) was incubated with ovalbumin, hsp110, or hsc70 at 43 °C for 30 min and diluted to 15 nM into 60% rabbit reticulocyte lysate (RRL; Promega) or hsc70 (1.6 μM) and Hdj-1 (3.2 μM) in a buffer containing 25 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, and 2 mM ATP (total, 50 μl) at 30 °C. For the measurement of luciferase activity, the reactivation solution was 5-fold-diluted in 25 mM Hepes, pH 7.5, and 10 μl was added to luciferase assay solution (Promega); the activity was measured with Lumat LB 9501 (Berthoid).

Survival Assay—Hamster hsp110-transfected Rat-1-R12 cells (described above) were grown in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and were induced to overexpress hsp110 by removal of 2 ng/ml doxycycline. Cells were cultured in the absence of doxycycline for 2 days with fresh changes of doxycycline-free media twice a day to remove residual drug. After indicated heat exposures, cells were counted and plated at different dilutions. After 10–14 days, colonies were stained with methylene blue and counted. The uninduced cells were treated identically, except doxycycline was maintained in the culture media. The presence or absence of doxycycline had no effect on the heat sensitivity of the parental Rat-1 cells. HeLa tetracycline-dependent transactivator 1 cells (HtTA-1, described above) were cultured and treated as described for Rat-1 cells.

RESULTS

To assess the role of hsp110 *in vivo*, we determined the effect of its overexpression on the long-recognized phenomenon of thermotolerance. For this purpose, we established two tissue culture cell lines (HeLa and Rat-1) in which the expression of hsp110 can be selectively controlled by a tetracycline-regulated expression system ("tet-off") in which removal of tetracycline induces its gene expression (12). Fig. 1*A* shows the expression of hamster hsp110 (induced), with a Myc-tag added to the carboxyl end in the control and heated Rat-1 cells using this system. In the *top panel*, an antibody reactive with both hamster and rat hsp110 demonstrates the expression of these hsp110 proteins in control and heated Rat-1 cells, whereas in the *bottom panel*, an anti-Myc antibody demonstrates the expression of hamster hsp110 in control cells. In these *in vivo* expression studies, the inducible level of hamster plus rat hsp110 in control cells was approximately comparable to the level of induction of rat hsp110 alone after heat exposure (*i.e.* is physiological). The expression data from the HeLa line were comparable. Hamster hsp110-induced cells were then challenged with potentially lethal heat doses, following which, the number of surviving cells was determined by clonogenicity. The effect of overexpression of hsp110 on the resistance of Rat-1 cells and HeLa cells is presented in Fig. 1, *B* and *C*, respectively. It is evident that cells containing exogenous hsp110 were significantly more resistant to heat killing. For comparative purposes, a survival analysis of fully thermotolerant cells arising from a conventional pre-heat treatment is also presented. Overexpression of hsp110 alone is capable of achieving approximately 25–33% full thermotolerant effect (the survival for uninduced Rat-1 cells at the later time point fell below the level of detection). This indicates that in the absence of overexpression of other hsps, hsp110 is still effective in protecting cells against potentially lethal heat exposures.

To better understand how hsp110 may protect cells from thermal shock *in vivo*, we determined the characteristics of this protein *in vitro* by utilizing previously applied assays for the analysis of other heat shock proteins and molecular chaperones. For this purpose, we purified histidine-tagged hsp110 from *E. coli* to homogeneity. Since hsp110 shares sequence

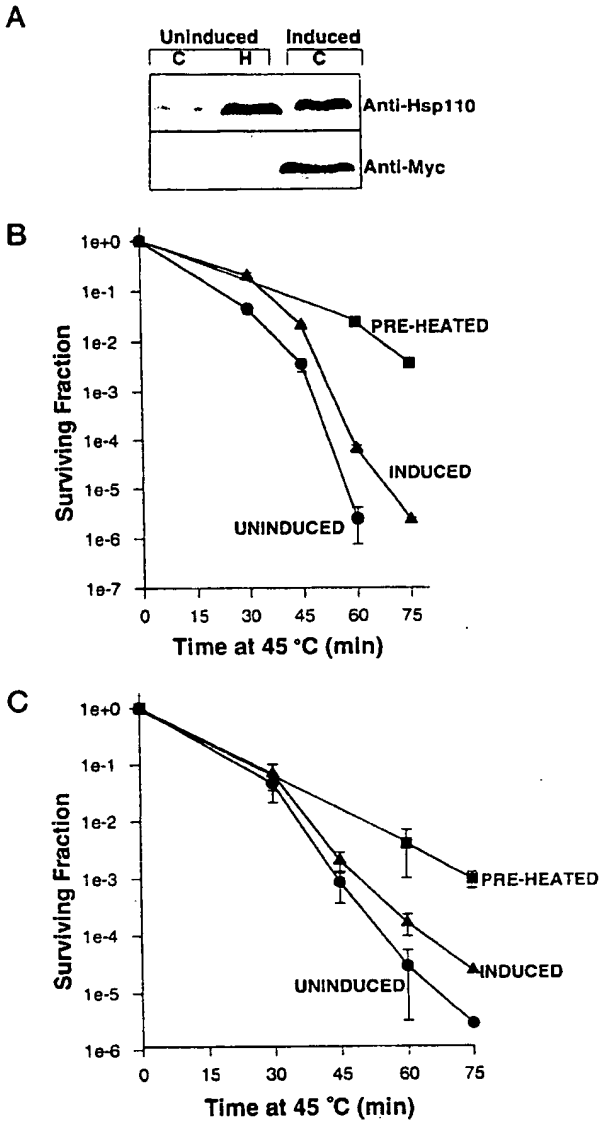

FIG. 1. **The effect of hsp110 overexpression on thermoresistance.** Hamster hsp110 was placed on a tet-off-inducible promoter. The expression of hsp110 in Rat-1 cells was then assessed as indicated in *panel A* using an antibody reactive with both exogenous and endogenous hsp110 (anti-hsp110, *top*) and with Myc-tagged hamster hsp110 only. The effect of control (*C*) and heat shock (*H*) on uninduced (no expression of hamster hsp110) is shown together with the expression hamster hsp110 plus endogenous hsp110 without heat. Cell cultures were then heated as indicated, and the percentage of surviving cells was determined by colony survival. The overexpression of hsp110 (induced) confers substantial thermoresistance to both Rat-1 (*panel B*) and HeLa (*panel C*) cells. In the case of Rat-1 cells, the longest time point yielded no viable colonies and was not plotted. The preheated curve indicates a standard thermotolerance curve for these cell lines resulting from a 45 °C, 15 min heat shock delivered 20 h before the challenge exposure.

similarities with the hsp70 family and since hsp70 proteins have been studied for their abilities to inhibit protein aggregation and promote protein folding (13–15), we similarly examined the ability of hsp110 to perform these chaperoning functions. For aggregation studies, we used luciferase and citrate synthase as model proteins. It is seen in Fig. 2*A* that hsp110 is efficient in inhibiting the heat-induced aggregation of luciferase *in vitro*. Most notably, hsp110 was nearly totally effective in inhibiting aggregation as assayed by light scattering when present in a 1:1 molar ratio. This suggests that the interaction

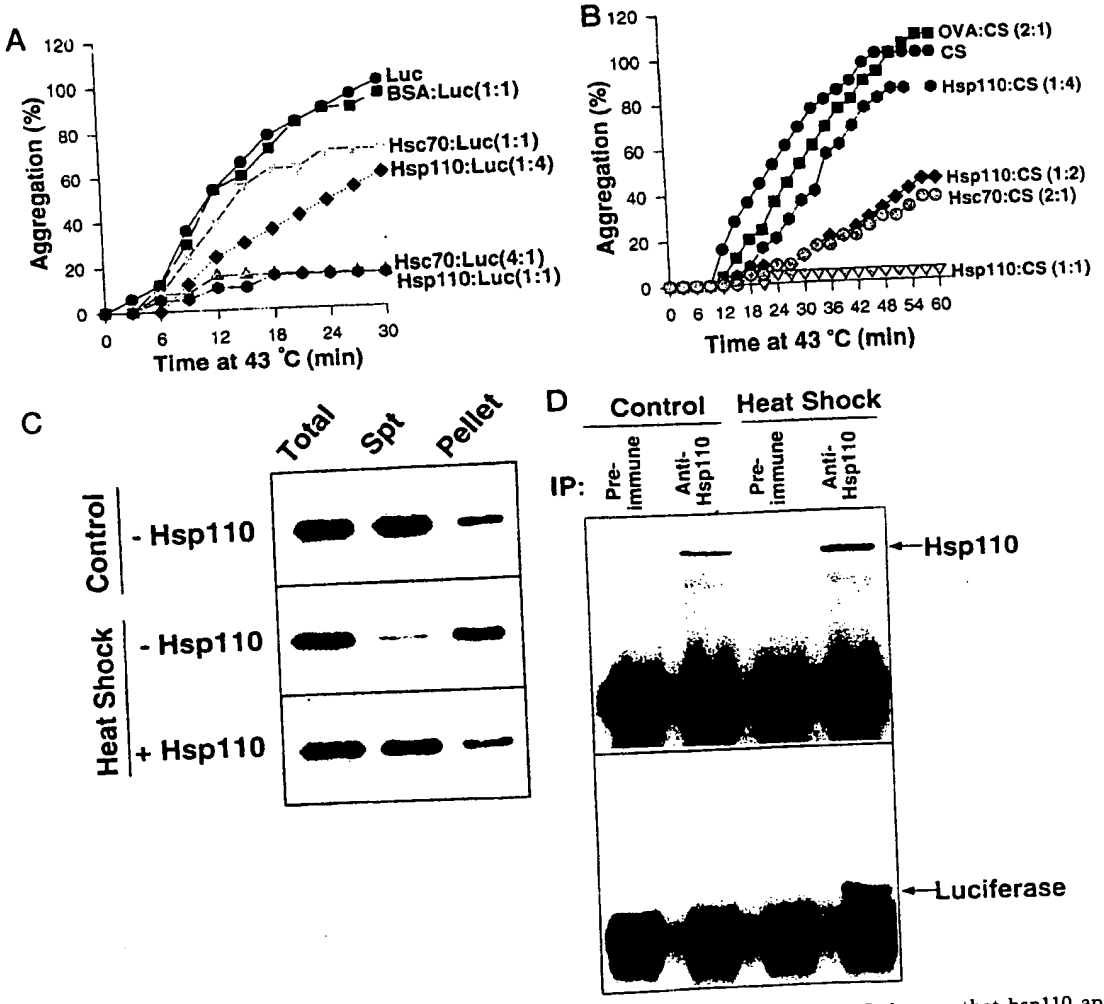

FIG. 2. **The effect of hsp110 and hsc70 on the inhibition of protein aggregation *in vitro*.** It is seen that hsp110 and hsc70 inhibit luciferase (Luc, *panel A*) and citrate synthase (*CS*, *panel B*) aggregation and that hsp110 performs this function significantly more efficiently than does hsc70. Bovine serum albumin (*BSA*) and ovalbumin (*OVA*) were used as control proteins. Molar ratios are indicated in *parentheses* at *right*. *Panel C* indicates the Western blot analysis of luciferase in the supernatant (*Spt*) and pellet of control and heat-shocked luciferase with and without hsp110 present. *Panel D* shows a coimmunoprecipitation (*IP*) analysis using antibody against hsp110 followed by Western blotting and probing with antibody against luciferase. This demonstrates that antibody against hsp110 coprecipitates heated luciferase but not Luciferase incubated at room temperature.

of one hsp110 to one denatured luciferase protein was sufficient to maintain solubility as measured in this way. For comparitive purposes, the efficiency of hsc70 in this process was examined in parallel. Consistent with some earlier studies (13) but not others (15), hsc70 was also capable of inhibiting the protein aggregation. However, in this case, total suppression of luciferase aggregation requires the association of 4 hsc70 proteins to 1 luciferase protein compared with the 1:1 ratio obtained with hsp110. Heat-induced aggregation studies using citrate synthase (shown in Fig. 2*B*) provide very similar data to that presented in Fig. 2*A* with luciferase. Therefore, in this important molecular chaperoning characteristic, hsp110 functions in a manner similar to hsc70 but is far more efficient in performing this molecular process than is hsc70.

The effect of hsp110 on luciferase aggregation as presented in Fig. 2*A* was also examined by Western blotting analysis using an antibody against luciferase. Fig. 2*C* demonstrates that whereas some freshly prepared luciferase is insoluble (pellet) under control conditions, most of the enzyme remains in the supernatant (*Spt*). However, when heated in the presence of bovine serum albumin, most luciferase is seen to become insoluble (*-hsp110*). If hsp110 is present during heating at a 1:1 molar ratio with luciferase (*+hsp110*), it is clear that the enzyme remains maximally soluble. In these studies, the presence or absence of ATP had no effect on the outcome, despite the fact that hsp110 contains the consensus sequences for ATP binding characteristic of this family of ATP binding proteins (4, 16).

To verify that this protective effect of hsp110 was due to its direct interaction with luciferase, we precipitated hsp110 and examined the coimmunoprecipitation of luciferase. As seen in Fig. 2*D*, luciferase coprecipitates with hsp110 (*bottom panel*) only when heated but not when the two proteins were incubated at room temperature. Moreover, luciferase was not coimmunoprecipitated with hsp110 when it was incubated with pre-heated hsp110 at room temperature (data not shown). Therefore, these experiments demonstrate that hsp110 selectively recognizes denatured proteins and prevents their aggregation during heat shock *in vitro*. That these proteins are heat-denatured is indicated by their loss of enzymatic activity. Additionally, whereas hsp110 inhibited aggregation, it had no effect on the rate of loss of this enzyme activity, nor was activity

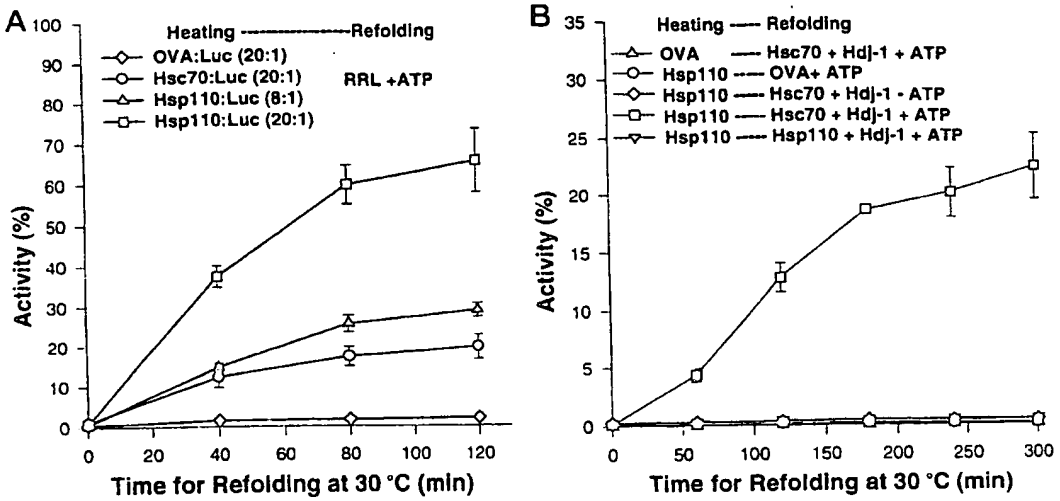

FIG. 3. **Luciferase bound to hsp110 can be refolded by rabbit reticulocyte lysate (*RRL, panel A*) or by hsc70 + Hdj-1 (*panel B*).** The protein present during the heating period and its molar ratio to luciferase is indicated. In *B*, the molar ratios during heating were 20:1 in each case. Ovalbumin (*OVA*) was used as a control protein. Whereas hsp110 sustains luciferase in a folding-competent state after heat shock, it cannot refold this protein either alone or in combination with Hdj-1.

regained by further incubations of the hsp110·luciferase complex at room temperature (data not shown). Thus, hsp110 is a potent chaperone in inhibiting aggregation but is incapable of refolding of heat-denatured proteins on its own.

This suggests that hsp110 would require the cooperation of other hsps and/or chaperones to refold denatured substrate proteins. To initially address this question, luciferase was heated in the presence of ovalbumin, hsc70, or hsp110 and then added to 60% RRL that has been shown to be an optimal refolding medium (17, 18). As seen in Fig. 3*A*, luciferase heated in the presence of hsp110 regained 70% original activity, whereas luciferase heated with hsc70 regained only 20% original activity (at the same molar ratio, 20 × hsp110 or hsc70 to 1 × luciferase). It is clear from this data that optimal folding requires significant additional hsp110 or hsc70 compared with optimal maintenance of solubility (Fig. 2). This demonstrates that when bound to hsp110, denatured luciferase is maintained in a folding competent state.

Since hsc70 and Hdj-1 alone have been shown to be a folding ensemble, we repeated the above RRL refolding assay using only these specific molecular chaperones. Although less efficient than RRL, Fig. 3*B* demonstrates that hsp110 functionally interacts with hsc70·Hdj-1 to refold heat-denatured luciferase to 25% original activity. Whereas ATP is not required for inhibition of luciferase aggregation, it is necessary for the refolding functions of RRL or hsc70·Hdj-1 as has been demonstrated previously (13–15). Lastly, when the refolding step employs hsp110 (and not hsc70) and Hdj1, no recovery of luciferase activity is obtained when the holding step also uses hsp110 (Fig. 3*B*). However, when the holding step (*i.e.* during heating) employs hsc70, a small amount of refolding is achieved by adding hsp110 and Hdj-1, probably reflective of the interaction of hsc70 (already present) and Hdj-1. This indicates that (i) hsp110/hsc70·Hdj-1 is a relevant protein folding machine, (ii) that based on Figs. 2, *A* and *B*, and Fig. 3*A*, hsp110 is notably more efficient than is hsc70 in protecting the denatured protein during the initial heating phase for subsequent refolding and iii), that Hdj-1 does not function as a folding co-chaperone with hsp110.

DISCUSSION

hsp110 was one of the earliest heat shock proteins described in mammalian cells and has been noted in numerous studies (*e.g.* Refs. 1–3). In Chinese hamster cells, hsp110 accounts for 0.7% total cell protein after heat shock compared with 3.2% for hsc70 + hsp70 and 1.2% for hsp90 (1). Its expression levels in the Rat-1 and HeLa cells are less distinctive than in Chinese hamster cells; however it remains as one of the major heat-inducible proteins in these cell lines. Its constitutive expression in different murine tissues varies widely, with highly significant levels of expression in liver and brain (4, 6). Indeed, the expression of hsp110 in brain is comparable to heat-shocked Chinese hamster cells (*i.e.* making it approximately 0.7% total brain mass). Additionally, like the hsp70 family, the hsp110 family possesses at least three distinct members, each of which is approximately 60% identical in amino acid sequence to the other. These are hsp110, apg-1 (osp94), and apg-2 (9–11). The cell and tissue expression of apg-1 and apg-2 is less well characterized than hsp110, although in testis and in renal medullary duct cells, apg-1/osp94 has been shown to be highly expressed (9, 11). The level of expression of hsp110 and its family members in different cell lines and in murine tissues speaks to a significant role for this class of stress proteins in both the stress response and in the normal functioning of the non-stressed cell.

The recent cloning of the cDNA for this protein from a variety of organisms as diverse as yeast and mammals has shown it to be a large and highly diverged relative of the hsp70 family (4–11, 29, 30). Secondary structure analysis of hsp110 demonstrates that it exhibits significant similarity to the secondary structure of hsp70 and DnaK, whose structures are well studied by several methods including crystallography. hsp70/DnaK is composed of 1) an amino-terminal ATP binding domain followed by 2) a 100-amino acid β-sheet region that has been identified as the peptide binding domain and 3) a carboxyl-terminal α-helical region that is involved in regulation of hsp70/DnaK function and appears as a lid covering the peptide binding domain (19). Analysis of the sequence and secondary structure of hsp110 demonstrates that it appears to have the same basic 1) ATP binding domain, 2) β-sheet (peptide binding) domain, and 3) carboxyl end α-helical region. However, the ATP binding domain of hsp110 binds and hydrolyzes ATP poorly relative to hsc70[2] (6, 16). The β-sheet configuration (*i.e.* the predicted peptide binding region of hsp110) shows some

[2] M. Murawski, H. Oh, and J. Subjeck, unpublished data.

sequence homology to corresponding regions in the structure of DnaK and appears to be of similar overall size and organization.[3] The regulatory, α-helical "lid" region of hsp110 is similar to that of DnaK but is larger than the corresponding region in DnaK. The predominant structural difference between hsp110 and DnaK/hsp70 is that the α-helical lid region of hsp110 is connected by a 100-amino acid "loop" to the β-sheet peptide binding domain, a structure that is virtually absent in hsp70 (4) and that is responsible for much of the increased size of hsp110. If the α-helical lid plays an important role in peptide binding as suggested (*cf.* Ref. 19), the interposition of this 100-amino acid loop between it and the peptide binding domain may have significant functional implications that could be related to the differences between hsp110 and hsc70 described here. It is also in the predicted α-helical lid region of hsp110 that the conserved sequences, which act as a signature for members of the hsp110/SSE family, reside (4).

The data presented here demonstrate that hsp110 must be added to the list of identified molecular chaperones that can inhibit the aggregation of denatured cellular proteins, a list that includes the small heat shock proteins, hsp90, the immunophilins, p23, as well as members of the hsp70 family (13, 20–24). However, it is evident from both the aggregation studies of luciferase and citrate synthase (Fig. 2) and from the refolding studies of luciferase with reticulocyte lysate (Fig. 3) that hsp110 is significantly more efficient in maintaining heat-damaged proteins in a soluble, refoldable state than is its evolutionary relative, hsc70. Interestingly, earlier reports are contradictory concerning the ability of mammalian hsp70 to hold denatured protein *in vitro*. Minami *et al.* (15) report that hsc70 alone does not inhibit aggregation of luciferase, whereas hsc70 plus Hdj-1 does. Freeman and Morimoto (13) show that hsp70 and hsc70 maintain the solubility of β-galactosidase as indicated by native gel electrophoresis, an observation in agreement with the present report. It is also shown here that hsp110 cannot refold luciferase on its own or with the addition of Hdj-1, a well known co-chaperone for folding with hsc70. However, the refolding obtained with rabbit reticulocyte lysate was much faster and higher than by hsc70 and Hdj-1, suggesting that other components in this lysate are required for efficient refolding of the denatured proteins. It is likely that other chaperones and co-chaperones exist, one or more of which could interact in protein folding with hsp110, possibly including a DnaJ homolog other than Hdj-1. Whereas the structural and sequence similarities between hsp110 and hsc70 indicate a similarity in function between these proteins, hsp110 could also play a primary role in the folding pathway without being directly involved in the final folding step. Furthermore, a molecular interaction between hsc70 and hsp110 has been observed in all cell lines and mouse tissues examined as indicated by coimmunoprecipitation studies and can be reproduced *in vitro*.[4] This suggests that in many instances at least, hsp110 may not function independently of hsc70 (and *vice versa*) and that the true *in vivo* physiologic function of these chaperones requires a cooperative interaction with substrate proteins, perhaps utilizing the different peptide binding capacities and ATP binding and hydrolysis characteristics of each. Indeed, since hsp110 is a prominent mammalian hsp (as it also appears to be in other organisms), it may play a primary role for holding the unfolded peptide chain, with hsc70 playing a secondary function in this initial chaperoning step.

That these *in vitro* chaperoning functions have a physiologic role *in vivo* is indicated by the thermoresistance/thermotolerance studies described in this report. A partial but significant degree of heat resistance is obtained by the overexpression of hsp110 in both HeLa and Rat-1 cells as determined by the cell ability to continue to proliferate and form colonies after severe heat exposures. This strongly suggests that the *in vitro* properties described above are operative *in vivo*.

Lastly, the hsp70 family has been shown to contain different cellular compartmental members with grp78 (or BiP) representing the endoplasmic reticulum representative. Likewise, hsp110 has a strong structural homolog resident in the endoplasmic reticulum known as grp170 (16, 25–28), although this stress protein may also exhibit its own independent properties. Multiple compartmentalization of these large and greatly diverged hsp70-related proteins further underscores the physiological importance of this family, and further studies of hsp110 and grp170 are necessary to understand the full complement of major molecular chaperones and how they interact in mammalian cells.

Acknowledgments—We are deeply indebted to Dr. Brian Freeman for supplying the purified Hdj-1 and for his valuable comments. We appreciate the comments and suggestions of Dr. Douglas Easton in the preparation of this manuscript as well as the technical expertise of Ms. Melanie Murawski.

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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 230, 94–99 (1997)
ARTICLE NO. RC965890

Cloning and Expression of cDNA Encoding the Human 150 kDa Oxygen-Regulated Protein, ORP150

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Received November 25, 1996

We have cloned a cDNA encoding the human 150 kDa oxygen-regulated protein (ORP150) from hypoxia-treated astrocytoma U373 cDNA library. The deduced amino acid sequence of 999 residues contains a signal peptide and an ER retention-like signal at the N- and C-termini, respectively. It has a striking sequence similarity (91% identity) with Chinese hamster 170 kDa glucose-regulated protein (GRP170). The N-terminal half of ORP150 exhibits significant similarity to the ATPase domain of HSP70 family proteins with well-conserved ATP binding motifs. Northern blot analysis revealed that induction of ORP150 in U373 cells was not limited to hypoxia but also observed by 2-deoxyglucose or tunicamycin treatment. Furthermore, tissue specificity of expression of ORP150 was quite similar to that of GRP78. These findings suggest that ORP150 participates in quality control of proteins in the ER in response to diverse environmental stresses. © 1997 Academic Press

Astrocytes, the most abundant cell type in mammalian brain, play an important role in the maintenance and regeneration of neuronal functions. They retain cell viability even in extreme ischemia and proliferate in damaged brain, leading to tissue remodeling in the central nervous system (1, 2). Rat astrocytes exposed to hypoxia followed by reoxygenation were reported to release increased amounts of interleukin 6 (IL-6) that could promote neuronal survival in ischemic brain (3). On the other hand, induction of a set of stress proteins with molecular masses of about 28, 33, 78, 94, and 150 kDa was observed in cultured rat astrocytes exposed to hypoxia or hypoxia/reoxygenation (4). The 78 kDa protein identified as GRP78 (BiP) was found to be involved in the hypoxia-induced production of IL-6 in astrocytes (4). The 94 kDa protein whose expression was also induced by 2-deoxyglucose treatment may correspond to GRP94. Although other proteins remain to be characterized, these observations raise the possibility that the induction of these proteins by hypoxia is implicated in the astrocyte capacity to proliferate and generate neurotrophic mediators under environmental perturbation such as brain ischemia.

In the previous study (5), we observed induction of a 150 kDa protein in the endoplasmic reticulum (ER) of cultured astrocytes specifically by hypoxia and not by other stimuli including heat shock, hydrogen peroxide, cobalt chloride, 2-deoxyglucose, or tunicamycin. The selectivity in response to oxygen deprivation led us to designate this protein ORP150 (150 kDa oxygen-regulated protein). Furthermore, cerebral ischemia in mouse caused induction of ORP150 in the ischemic area (5), suggesting that induction of ORP150 is part of central nervous response to oxygen deprivation.

Here we report the cloning of human and rat ORP150 cDNAs from hypoxia-treated human astrocytoma U373 cells and rat astrocytes, respectively. Comparison of the predicted amino acid sequences encoded by the cDNAs with protein data bank revealed that ORP150 most likely represents human and rat homologues of Chinese hamster GRP170, recently reported to be a member of large and highly diverged class of HSP70-like proteins (6). Northern blot analysis revealed a marked similarity between ORP150 and GRP78 in stress inducibility in U373 cells and tissue specificity of expression.

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Abbreviations: ER, endoplasmic reticulum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GRP, glucose-regulated protein; ORP, oxygen-regulated protein; UTR, untranslated region.

0006-291X/97 $25.00

MATERIALS AND METHODS

Cell culture and conditions for hypoxia. Astrocytes prepared from neonatal rats were cultured and exposed to hypoxia as described previously (5). Human astrocytoma U373 cells obtained from American Type Culture Collection were grown in Dulbecco's Modified Eagle Medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum. Cultures which achieved 70% confluence were exposed to hypoxia using an incubator attached to an hypoxia chamber (Coy Laboratory Products, Ann Arbor, MI) (7).

Amino acid sequence analysis of rat ORP150. Rat ORP150 was purified from cultured rat astrocytes exposed to hypoxia for 48 h and transferred to a polyvinyllidene difluoride (PVDF) membrane as described (5). The band corresponding to ORP150 was cut out and subjected to sequence analysis using a Model 476A sequencer (Perkin-Elmer Corp.), yielding a unique N-terminal 31 amino acid sequence, LAVMSVDLGSESMKVAIVKPGVPMEIVLNKE. The underlined sequence was used to design a degenerate PCR primer.

Construction and screening of cDNA libraries. Total RNA was isolated from hypoxia-treated rat astrocytes or U373 cells with acid guanidinium-phenol-chloroform method (8), and poly(A) RNA was purified by using oligo(dT)-magnetic beads BioMag$(dT)_{20}$ (PerSeptive Diagnostics, Cambridge, MA). Double stranded cDNA was synthesized with random hexamer and oligo(dT) primers by using a SuperScript Choice System kit (Life Technologies, Inc.) and was ligated to the EcoRI site of pSPORT1 (Life Technologies, Inc.) to construct a cDNA library. First, a 480 bp fragment encoding a part of rat ORP150 was obtained from the rat cDNA library by PCR using 5'-AARCCIGGIGTNCCNATGGA-3' and 5'-AATACGACTCACTATAGGGA-3' as primers. The former degenerate oligodeoxynucleotides encode KPGVPME found in the N-terminal sequence, and the latter corresponds to the antisense strand of the T7 promoter region in pSPORT1 vector. Second, the rat astrocytes cDNA library was probed with this 480 bp fragment, and a positive clone containing a 2,800 bp insert lacking the 3' region of the coding sequence was obtained. Finally, to clone human ORP150 cDNA, the human U373 cDNA library was screened with 5'-GCACCCTTGAGGAAAATGCT-3' (complementary to nucleotides 2699-2718 of rat 2,800 bp cDNA) as a probe by using a GeneTrapper cDNA Positive Selection kit (Life Technologies, Inc.) according to the manufacturer's instruction. The rat cDNA library was also screened in the same manner to obtain a cDNA encompassing the entire coding region. Nucleotide sequencing was carried out in both directions by a primer-walking strategy.

Northern blot analysis. A 4.5-kb EcoRI fragment of human ORP150 cDNA was labeled with [α-^{32}P] dCTP (3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) by using a DNA labeling kit (Pharmacia), and used as a hybridization probe. 20 μg of total RNA prepared from U373 cells exposed to various stresses were electrophoresed and transferred onto a Hybond N^+ membrane (Amersham Corp.). Multiple Tissue Northern Blots, in which each lane contained 2 μg of poly(A) RNA from the adult human tissues indicated, was purchased from Clontech. The filter was hybridized at 65 °C in the Rapid-hyb buffer (Amersham Corp.) with human ORP150, GRP78, glyceraldehyde-3-phosphate dehydrogenase (G3PDH), and β-actin cDNAs each labeled with [α-^{32}P] dCTP, washed with 0.1 × SSC containing 0.1% SDS at 65 °C, and followed by autoradiography.

RESULTS AND DISCUSSION

Cloning of ORP150 cDNA

Since the reported N-terminal 15 amino acid sequence of rat ORP150 (5) was not appropriate for designing mixed probes or primers because of large degeneracy, we further performed a sequence analysis of purified rat ORP150 to determine additional 16 residues. Based on this newly determined amino acid sequence, we first cloned rat ORP150 cDNA, which should facilitate cloning of the human counterpart.

To obtain a specific probe for cloning rat ORP150 cDNA, a cDNA library prepared from cultured rat astrocytes that had been exposed to hypoxia was subjected to PCR. The amplified 480 bp fragment thus obtained was sequenced and found to contain a 39 bp nucleotide sequence encoding part of the N-terminal sequence KPGVPMEIVLNKE. Northern blot analysis using this fragment as a probe revealed that mRNA with a length of about 4 kb was induced in cultured rat astrocytes upon hypoxia (data not shown). The rat cDNA library was then screened with this PCR fragment as a probe, and a 2,800 bp cDNA containing most of the coding region but lacking the C-terminal portion was isolated. To obtain human ORP150 cDNA, a cDNA library prepared from hypoxia-treated U373 cells was screened by the Gene Trapper system. Briefly, the cDNA library was converted to single stranded DNA, and the desired cDNA clone was then enriched by hybridization with a 20-mer oligonucleotide derived from the 3' region of the 2,800 bp rat cDNA clone. A full-length 4,503 bp human cDNA was isolated and found to contain a 2,997 bp open reading frame predicted to encode a polypeptide of 999 amino acids with a calculated molecular mass of 111,330 Da (Fig. 1). The first ATG was thought to be the translation initiation site, since a TGA nonsense codon was located 99 bp upstream in the same frame and the flanking nucleotides matched Kozak's criteria (purine at position −3 and G at +4) (9). A polyadenylation signal (AATAAA) was found 20 bp upstream of the poly(A) tail. A 3,252 bp rat cDNA containing an open reading frame with the same length but lacking most of the 3' untranslated region was isolated in the same manner (the sequence is available from GenBank under accession number U41853).

We confirmed that the cloned cDNA encodes ORP150 by transient expression in COS-7 cells transfected with human ORP150 cDNA ligated into pSV-SPORT1 (Life Technologies, Inc.). Western blotting of the cell lysate using antibody raised against purified rat ORP150 (5) gave an immunoreactive band at a position of 150 kDa (data not shown). The deduced amino acid sequences of human and rat ORP150 exhibit high similarity (over 91% identity) to each other and to those of Chinese hamster GRP170 (GenBank U34206) for the entire molecule. Furthermore, amino acids 347-999 of human ORP150 showed 90% identity with the amino acid sequence of mouse ER-resident Ca^{2+}-binding protein CBP-140 deduced from partially cloned cDNA (10). Although ORP150, GRP170, and CBP-140 were identified from different species on different criteria, such high

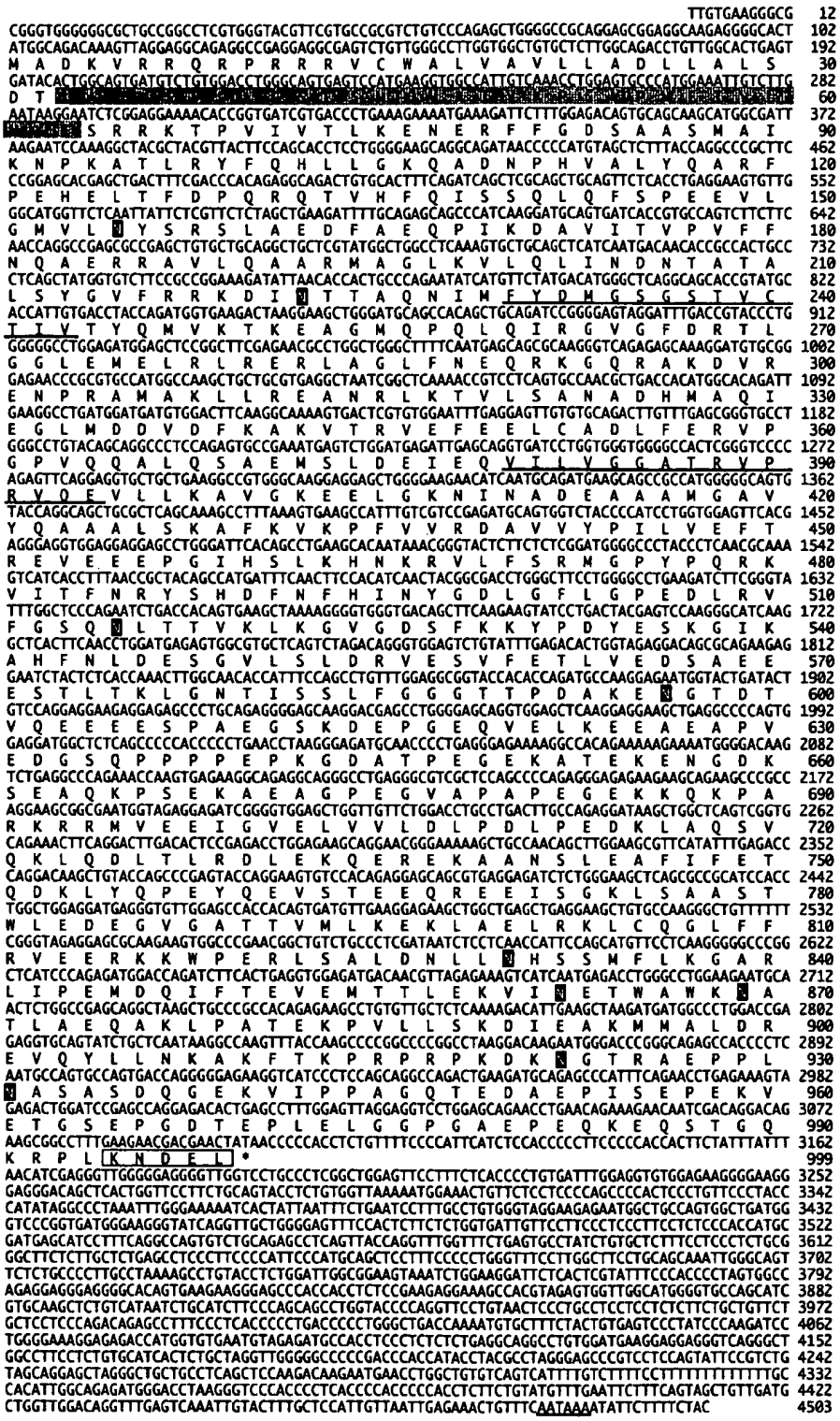

FIG. 1. Nucleotide sequence of human ORP150 cDNA and the predicted amino acid sequence. The top line shows the nucleotide sequence of the cDNA for human ORP150, and the second line shows the predicted amino acid sequence. The shaded amino acids are identical with the N-terminal 31 amino acid sequence of purified rat ORP150. The underlined amino acid sequences correspond to the HSP70 family signatures 2 and 3. The potential N-linked glycosylation sites are highlighted; the ER-retention signal-like sequence at the C-terminal is boxed. The nucleotide sequence underlined represents the potential polyadenylation signal. GenBank Accession Number is U65785.

similarities suggest that they represent functionally homologous (orthologous) proteins.

The nucleotide sequence similarity of the coding regions between human ORP150 and hamster GRP170 cDNAs was 88%, consistent with the striking conservation at the amino acid level. In contrast, both the 5′ and 3′ untranslated regions (UTRs) of these cDNAs exhibited marked diversities. The 3′ UTR of the human ORP150 cDNA (1,400 bp) was much longer than that of the hamster GRP170 cDNA (845 bp), and their overall sequence similarity was only 39%. The 5′ UTR of hamster GRP170 cDNA (54 bp) also exhibited only 44% similarity to the corresponding region of human ORP150 cDNA. Although UTR sequences are usually less conserved among species than coding sequences, such remarkable divergency suggests regulatory consequences.

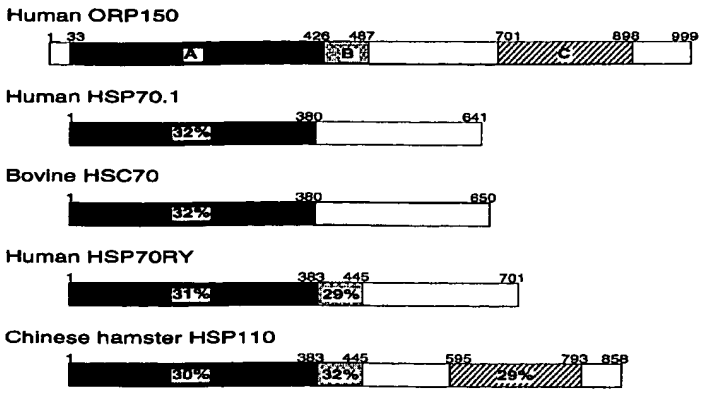

FIG. 2. Amino acid sequence similarities among human ORP150 and HSP70 family proteins. Region A containing the ATPase domain exhibits a modest similarity among HSP70 family proteins, and the similarity with HSP70RY and HSP110 further extends to region B. Similarity is also found for region C between human ORP150 and Chinese hamster HSP110. The first 32 amino acids of ORP150 represent the putative signal peptide.

Structural Features of ORP150

The N-terminal amino acid sequence obtained with purified rat ORP150 corresponded to amino acids 33-63 deduced from both the human and rat cDNAs, indicating that the first 32 residues represent the signal peptides for secretion. The C-terminal KNDEL sequence, which resembles KDEL sequence, a signal to retain the ER-resident proteins (11), may function as an ER-retention signal. The existence of a signal peptide at the N-terminus and the ER-retention signal-like sequence at the C-terminus suggests that ORP150 resides in the ER, consistent with the previous results of immunocytochemical analysis of rat hypoxic astrocytes (5). The deduced amino acid sequence contains nine potential N-linked glycosylation sites and more than 20 sites of phosphorylation by serine/threonine kinase, suggesting that at least part of the discrepancy between observed and calculated molecular masses of ORP150 is due to post-translational modifications such as glycosylation and phosphorylation.

Analysis of protein data bases with the BLAST program (12) showed that the N-terminal half of ORP150 has a modest similarity to the ATPase domain of numerous HSP70 family sequences. An extensive analysis with pairwise alignments (13) revealed that amino acids 33-426 of human ORP150 was 32% identical to amino acids 1-380 of both inducible human HSP70.1 (14) and constitutive bovine HSC70 (15), typical members of HSP70 family (Fig. 2). An additional region similar to HSP70RY and hamster HSP110, which both belong to a new subfamily of large HSP70-like proteins (16), extended further to residue 487. A protein sequence motif search with PROSITE (17) showed that ORP150 contains two of the three HSP70 protein family signatures: FYDMGSGSTVCTIV and VILVGGATRVPRVQE which completely matched with the HSP70 signatures 2 and 3, respectively (Fig. 1, underlined), and VDLG (amino acids 38-41) which matched with the first four amino acids of the signature 1. Furthermore, the N-terminal region of ORP150 contained a putative ATP-binding site consisting of the regions (amino acids 36-53, 197-214, 229-243, 378-400, and 411-425) corresponding to the five motifs specified by Bork et al. (18). Although the C-terminal putative peptide-binding domains of HSP70 family are generally less conserved (19), the C-terminal region flanked by nucleotides 701 and 898 shared appreciable similarity with HSP110 (amino acids 595-793; 29% identity).

Stress Inducibility of ORP150 mRNA

In view of the striking sequence similarity between ORP150 and GRP170 characterized as a glucose-regulated protein (20), we examined whether ORP150 mRNA was inducible by stresses other than hypoxia. Total RNAs prepared from astrocytoma U373 cells exposed to various stresses were analyzed by Northern blotting using 4.5 kb of human ORP150 cDNA as a probe. As shown in Fig. 3, the ORP150 mRNA level was highly enhanced upon 24-48 h of exposure to hypoxia. In parallel experiments, treatment with 2-deoxyglucose (25 mM, 24 h) or tunicamycin (5 μg/ml, 24 h) enhanced ORP150 mRNA to the levels comparable to that induced by hypoxia. The induction levels were also comparable with those observed for mRNA of a typical glucose-regulated protein GRP78. Heat shock treatment failed to enhance ORP150 mRNA appreciably.

Apparently, some of the environmental stresses that cause accumulation of unfolded proteins in the ER induced ORP150 mRNA in U373 cells. Similar induction was also observed in HeLa cells (data not shown). Thus, the induction of ORP150 is not restricted to hypoxia at

least in some established cell lines, in contrast to highly selective induction observed with cultured rat astrocytes (5) and human mononuclear phagocytes (21). This finding may be interpreted to mean that actively growing cells need a higher level of ORP150 expression to cope with perturbation of the ER functions caused by accumulation of unfolded or misfolded proteins.

Tissue Specificity of ORP150 Expression

Expression of ORP150 was studied by Northern blot analysis of poly(A) RNA prepared from eight different human tissues using ^{32}P-labeled ORP150 cDNA as a probe. ORP150 mRNA was found to be highly expressed in the liver and pancreas, whereas little expression was observed in kidney and brain (Fig. 4). Furthermore, the tissue specificity of ORP150 expression was quite similar to that of GRP78. The higher expression observed in the tissues that contain well-developed ER and synthesize large amounts of secretory proteins is consistent with the finding that ORP150 is localized in the ER (5).

In conclusion, both the characteristic primary protein structure and the similarity found with GRP78 in stress inducibility and tissue specificity suggest that ORP150 plays an important role in protein folding and secretion in the ER, perhaps as a molecular chaperone, in concert with other GRPs to cope with environmental stress.

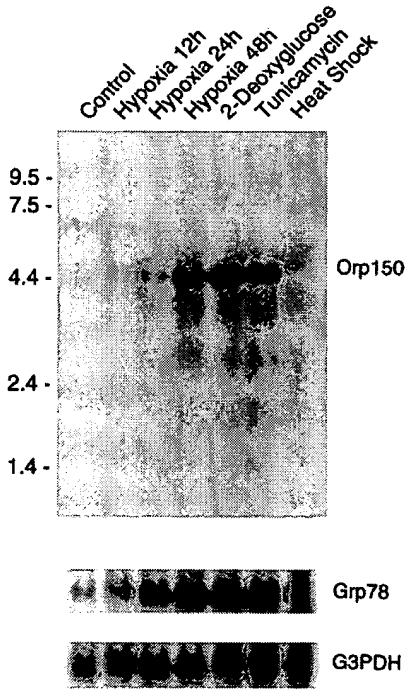

FIG. 3. Stress inducibility of ORP150 mRNA in human astrocytoma U373 cells. Total RNA was extracted and Northern blot analysis was performed using ^{32}P-labeled human ORP150 cDNA as a probe. Human GRP78 cDNA and G3PDH cDNA each labeled with [α-^{32}P] dCTP were rehybridized to the same filter. Hypoxia, treated for the indicated time period; 2-deoxyglucose, 25 mM for 24 h; tunicamycin, 5 μg/ml for 24 h; heat shock, at 43 °C for 1 h and recovery at 37 °C for 2 h. Sizes (in kilobases) are indicated on the left.

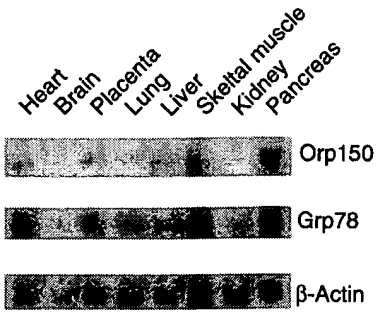

FIG. 4. Tissue specificity of ORP150 expression. Multiple Tissue Northern Blots (Clontech, Inc.), in which each lane contained 2 μg of poly(A) RNA from the adult human tissues indicated, were hybridized with ^{32}P-labeled human ORP150 cDNA. Human GRP78 cDNA and β-actin cDNA each labeled with [α-^{32}P] dCTP were rehybridized to the same filter.

ACKNOWLEDGMENTS

We thank Masako Nakayama and Mayumi Ueda for technical assistance.

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Heat shock proteins: A review of the molecular chaperones

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The emergence of sophisticated molecular biology methods has resulted in major advantages in the basic science knowledge of vascular diseases. However, this new knowledge often is poorly understood by our readers because the methods and mechanisms of molecular and cellular biology are complex and most developments have been made since their formal training was complete. In publishing invited reviews of some of the key issues in basic science, we aim to provide vascular surgeons with basic background information that is pertinent to the understanding of the underlying basic science.

Anton Sidawy, MD
K. Wayne Johnston, MD
Robert B. Rutherford, MD

Heat shock proteins are ubiquitous proteins found in the cells of all studied organisms. Many types of stress, including heat, induce expression of a family of genes known as the heat shock protein genes. Heat shock proteins originally were discovered when it was observed that heat shock produced chromosomal puffs in the salivary glands of fruit flies (*Drosophilia*). The DNA sequence that makes up this family of genes is highly conserved across species. This family of genes originally was named because of their expression after exposure to heat. However, the genes are now known to be induced by a wide variety of environmental or metabolic stresses that include the following: anoxia, ischemia, heavy metal ions, ethanol, nicotine, surgical stress, and viral agents. Thus, the term "heat shock protein" is a misnomer because many agents other than heat induce the expression of the heat shock protein gene. Consequently, "stress protein" is the preferred term. Stress proteins are critically important because they appear to be necessary in the critical step of three-dimensional folding of some newly formed proteins within the cell. In fact, they ensure that newly formed polypeptides proceed correctly through folding and unfolding to eventually achieve a functional shape (Fig 1). Stress proteins also assist in the repair of denatured proteins or promote their degradation after stress or injury. They have been referred to as "molecular chaperones" because of this function.

It is thought that stress proteins are produced in response to nonlethal stress to protect organisms from subsequent severe stress that would otherwise be lethal. In the case of exposure to heat, this phenomenon has been called "thermotolerance" and has launched many experiments in which an association has been found between the heat shock response and protection against other stresses, such as hypoxia or ischemia. The addition of one type of stress may provide protection against other types of insults, which results in cross-tolerance. As examples, stress protein induction by hyperthermia may provide protection during a subsequent arterial injury or exposure to a heavy metal may provide subsequent protection against heat or ischemic injury. This thermotolerance treatment strategy has proved successful in experimental models of cardiac ischemia, arterial injury, endotoxic shock, renal and hepatic ischemia, ethanol-induced gastric ulcerations, and skeletal muscle ischemia-reperfusion.

Stress proteins belong to a multigene family and range in molecular size from 8 to 150 kd (Table I). The nomenclature of these proteins and genes can be confusing because different nomenclature has been used in publications on this topic. The stress proteins typically are named according to their molecular size. The 70-kd protein is referred to as Hsp70, and the

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J Vasc Surg 1999;29:748-51.

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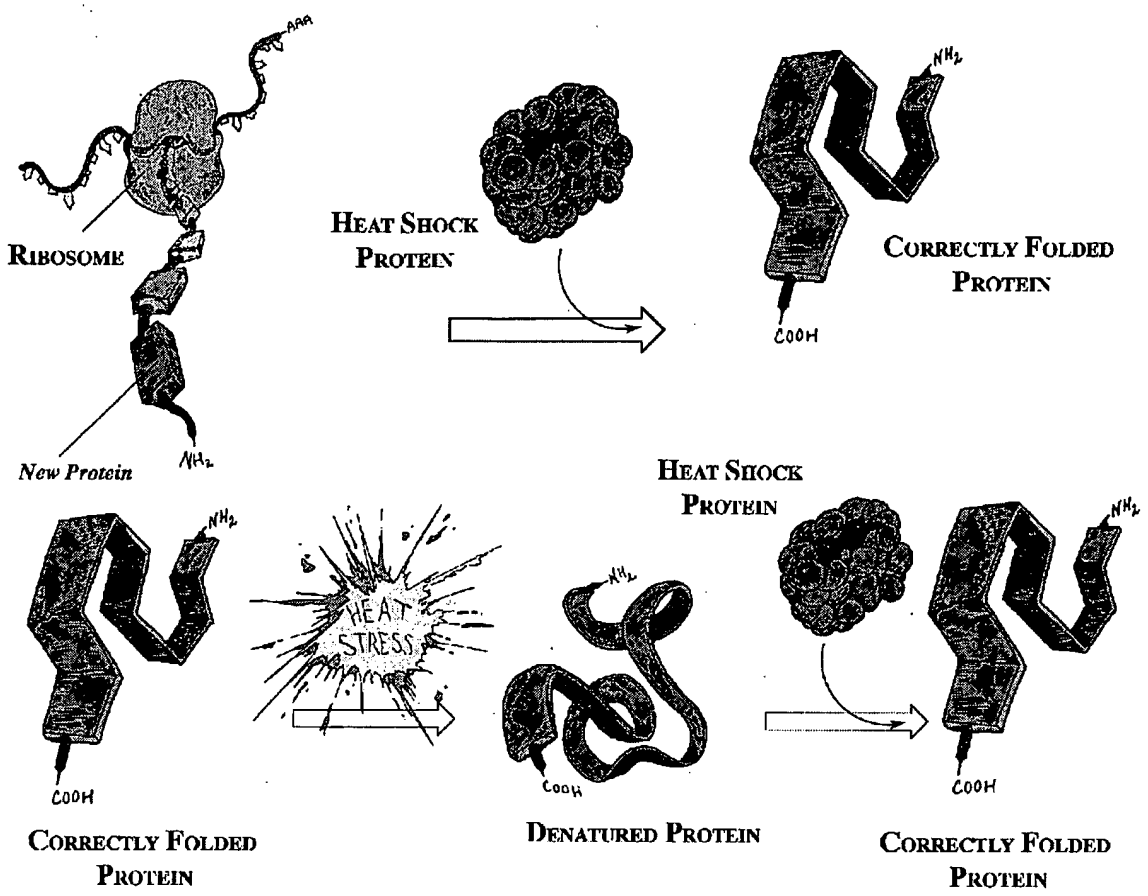

Fig 1. Two functions of heat shock proteins. *Top:* As new polypeptide chains (proteins) are being produced by ribosome within the cell, heat shock proteins assist in correct folding of polypeptide chain into functional protein. Presence of heat shock protein *(purple)* assures that the new protein will assume its functional three-dimensional configuration. *Bottom:* After stress event, heat shock proteins also assist in refolding or degradation of damaged or denatured proteins.

Table I. Stress proteins

Name	*Molecular size (kd)*	*Location*	*Remarks*
Ubiquitin	8	Cytosol/nucleus	Facilitates targeting and removal of proteins denatured by stress
Hsp10	10	Mitochondria	Cofactor for Hsp60
Low–molecular weight hsps	20 to 30	Cytosol/nucleus	Some may be responsible for regulating the cellular cytoskeleton and migration, and others regulate vascular tone and vessel wall remodeling
Hsp56	56	Cytosol	Binds and stabilizes the steroid hormone receptor complex
Hsp60	60	Mitochondria	Molecular chaperone
Hsp72	70	Cytosol/nucleus	Highly stress inducible (tolerance)
Hsp73	70	Cytosol/nucleus	Constitutively expressed molecular chaperone
Hsp90	90	Cytosol/nucleus	Part of the steroid receptor complex

gene coding for that protein would be hsp70. Many of the stress proteins are present continuously (constitutive expression), and expression of other proteins is increased by stress (stress inducible). Stress proteins are rapidly induced through transcription (messenger RNA production from DNA occurs in minutes) and translation (protein production from messenger RNA) mechanisms. Gene transcription is controlled by heat

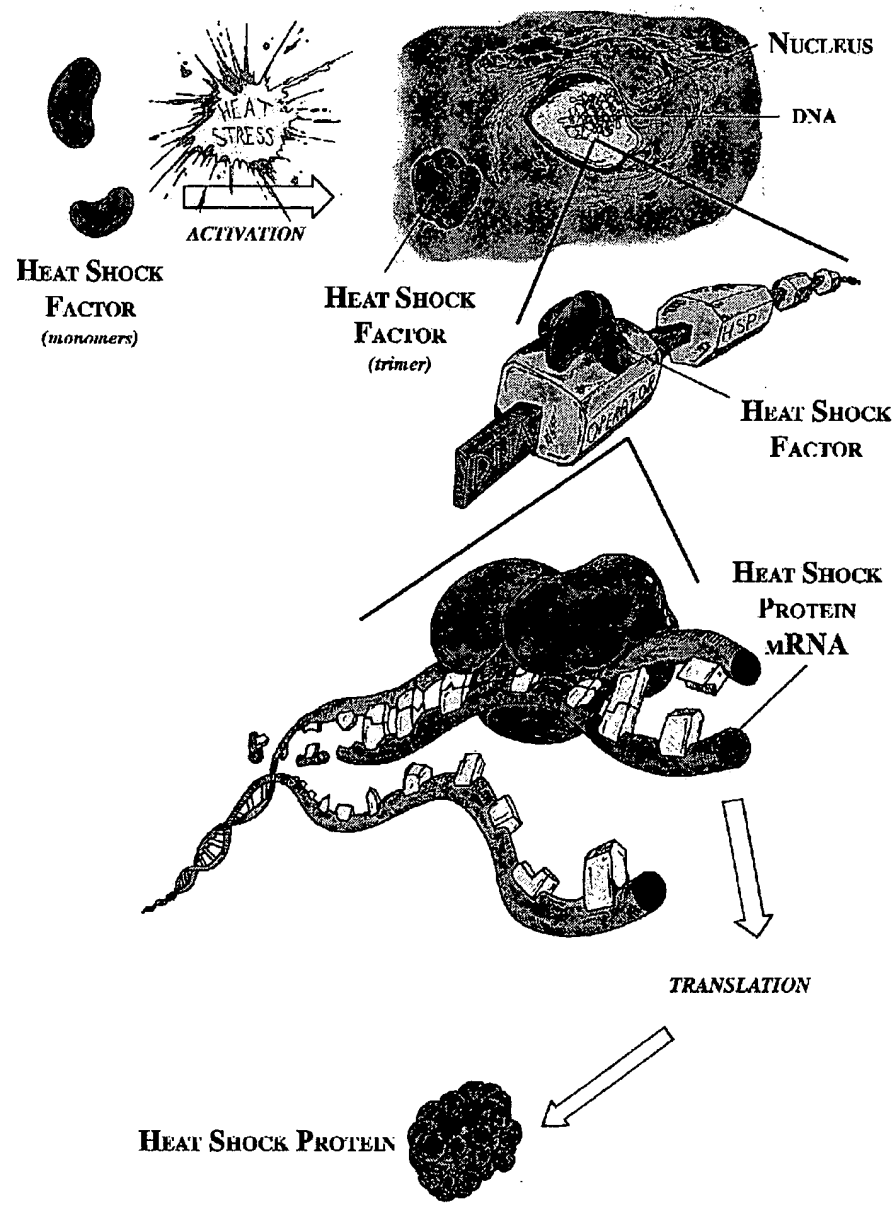

Fig 2. Upregulation of heat shock protein. Many types of stress are capable of increasing expression of some heat shock proteins (inducible). Stress results in activation of heat shock factor (HSF) monomers *(red)*. HSF monomer moves from cytosol to nucleus where it combines with other monomers to form a trimer. Trimer of HSFs attaches to operator (promotor site) on heat shock gene (DNA). Attachment of trimer to operator results in production of heat shock protein messenger RNA *(green)*, and transcription occurs in minutes. Messenger RNA moves to cytosol where heat shock protein is formed (translation).

shock transcription factors. Different members of the heat shock transcription factor family may be activated by specific stresses. Inactive heat shock factors exist as monomers. However, once activated, they trimerize into an active form that is capable of binding to the promotor site of the stress protein gene and initiating transcription and translation (Fig 2).

Abnormal levels of stress proteins have been found in a number of disorders, including atherosclerosis, congestive heart failure, fever, infection, aging,

Alzheimer's disease, malignant diseases, and autoimmune disorders. There is a growing body of evidence that some stress proteins may be associated with atherosclerosis. Experimentally, arteriosclerotic lesions can be induced by immunization with Hsp60/65. Hsp60/65 is found in high concentrations in human arteriosclerotic lesions, and there is a correlation between anti-Hsp60/65 antibodies and atherosclerosis. In addition to being involved in specific disease processes, the stress proteins may play a key regulatory role in cell death pathways (apoptosis) that involve DNA and protein synthesis. These proteins now are being implicated in the aging process. It appears that there is decreased expression of stress protein genes and decreased activity of HSF-1 during aging. These factors may make aging tissues more susceptible to oxidative stress injury.

Members of the Hsp70 family are the most extensively studied group of stress proteins to date. Some members of the Hsp70 family are expressed constitutively, and others are strictly stress inducible. The constitutively expressed protein shares about 95% sequence homology (identity of the DNA sequence) with the inducible form of Hsp70. However, little is known about its function within the cell. Upregulation of the inducible form of Hsp70 has been most closely associated with the development of thermotolerance.

The 90-kd (Hsp90) family of proteins represents one of the most abundant proteins in mammalian cells, yet its synthesis still increases after stress. It appears that Hsp90, in conjunction with Hsp70 and Hsp56, binds, stabilizes, and maintains the estrogen receptor complex in an active confirmation. Hsp90 appears to interact with multiple intracellular proteins and signal transduction pathways. Hsp90 serves a regulatory role by binding to and either inhibiting or stimulating the activity of its target protein. Thus, Hsp70 and Hsp90 are ubiquitous in all tissues, but some of the smaller stress proteins may have a more specialized role in the vascular system.

Hemeoxygenase (Hsp32), a rate-limiting enzyme in the degradation of Heme, is stress induced and is abundant in myocardial cells. Hsp32 is induced by sheer stress and may mediate nitric oxide–dependent platelet inhibition and vasodilatation. There is no direct evidence that Hsp32 functions as a chaperone, but its overexpression during stress events indicates that it may function in this fashion. Hsp25/27 influences the cell cytoskeleton (actin polymerization) and may be involved in cell migration. Physiologic stress increases the phosphorylation of Hsp27. Phosphorylation of Hsp25 occurs via the mitogen-activated protein kinases. Mitogen-activated protein kinases are involved in the intracellular signaling cascade and are activated during ischemia-reperfusion. Many of the small stress proteins that are present in the cytosolic compartment may be important in cardiovascular biology. The 20-kd protein, Hsp20, found in vascular smooth muscle, is a substrate for protein kinase and probably has a role in the maintenance of vascular tone and vessel wall remodeling. Ubiquitin is a small 8-kd stress protein that may facilitate targeting and removal of other proteins denatured during the stress event.

Stress proteins play a critical role in the maintenance of normal cellular homeostasis. These proteins almost certainly have a pivotal role in cell cycle progression and cell death (apoptosis) and are involved in many disease processes, including cardiovascular disease. Currently, the manipulation of stress proteins remains cumbersome because hyperthermia and pharmocologic manipulations are relatively nonspecific. Eventually, as we gain more insight into the exact role and function of these fascinating molecules, the clinical manipulation of the stress proteins will almost certainly prove beneficial.

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Submitted Feb 22, 1999; accepted Feb 22, 1999.

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Heat shock protein vaccines against cancer.

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Vaccination of mice with heat shock proteins (HSPs) derived from a tumor makes the mice resistant to the tumor from which the HSP was obtained. This phenomenon has been demonstrated with three HSPs--gp96, hsp90, and hsp70. Vaccination with HSPs also elicits antigen-specific cytotoxic T lymphocytes (CTLs). The specific immunogenicity of HSPs derives apparently, not from the HSPs per se, but from the peptides bound to them. These observations provide the basis for a new generation of vaccines against cancer. The HSP-based cancer vaccines circumvent two of the most intractable hurdles to cancer immunotherapy. One of them is the possibility that human cancers, like cancers of experimental animals, are antigenically distinct. The prospect of identification of immunogenic antigens of individual cancers from patients is daunting to the extent of being impractical. The observation that HSPs chaperone antigenic peptides of the cells from which they are derived circumvents this extraordinary hurdle. Second, most current approaches to cancer immunotherapy focus on determining the CTL-recognized epitopes of cancer cell lines. This approach requires the availability of cell lines and CTLs against cancers. These reagents are unavailable for an overwhelming proportion of human cancers. In contrast, the HSP-based vaccines do not depend on the availability of cell lines or CTLs nor do they require definition of the antigenic epitopes of cancer cells. These advantages, among others, make HSPs attractive and novel immunogens against cancer.

Good motivation

PMID: 8280719 [PubMed - indexed for MEDLINE]